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NAME OF AUTHOR/*NOM DE L'AUTEUR* Sherman D. Nelson

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NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. J. M. Mayo

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PERMANENT ADDRESS/*RÉSIDENCE FIXE*
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THE UNIVERSITY OF ALBERTA

STOMATAL PHYSIOLOGY OF *PAPHIOPEDILUM SPP.*

by

Ⓒ SHERMAN D. NELSON

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Stomatal Physiology of *Paphiopedilum spp.*" submitted by Sherman D. Nelson in partial fulfilment of the requirements for the degree of Master of Science.

James M. Mayo
Supervisor

John Hoddinott

David D. Cass

Kenneth D. Higginbotham

Date..Sept. 9/76..........

ABSTRACT

The stomatal physiology of hypostomatous lady slipper orchids *(Paphiopedilum spp.)* particularly *P. leeanum* was studied. This species was found to exhibit 'normal' stomatal responses in that guard cells opened in the light, closed in the dark, showed greater stomatal opening in treatments of blue rich *vs* red rich light and were effected by changes in [CO_2]. The rate of stomatal response was found to be similiar to that of other species reported in the literature. Abaxial leaf resistances when stomata were closed were generally found to be greater than 50 sec cm^{-1} and when open generally less than 30 sec cm^{-1}.

However, microscopic observation at the light level (fluorescence microscopy) and at the electron microscope level showed that the guard cells as well as other epidermal cells were non-chlorophyllous. This study constitutes the first report in the literature of a species which exhibits normal stomatal response and a non-chlorophyllous epidermis. Evidence is presented to indicate that K^+ is not involved as the major osmoticum in the stomatal movements of these species. Again this is the first report in the literature of such a condition where 'normal' stomatal responses are exhibited. As the meso-

phyll contains normal amounts of K^+ process from the epidermis is suggested. A relationship between the non-chlorophyllous condition and the potassium deficiency of the epidermis is suggested.

It would appear, based on the evidence presented in this thesis, that a unique stomatal mechanism is operating in *Paphiopedilum spp.* or that a re-evaluation of present theories is required.

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INTRODUCTION

This thesis is a study of stomatal physiology in leaves of various lady's slipper orchids in the genus *Paphiopedilum*, particularly *P. leeanum*. The study involves both anatomical and physiological investigations. The concluding section of the thesis attempts to integrate the results and point out the important implications of the work.

The central question of the thesis concerns the necessity of guard cell chlorophyll in stomatal operations. Ontogenetic studies (Frommhold, 1971; Reuter, 1942) and studies with etiolated seedlings (Virgin, 1956) indicate that the start of 'normal' stomatal functioning corresponds to the occurrence of either detectable levels of guard cell chlorophyll or well developed guard cell chloroplasts. Both Shaw (1958) and Virgin (1957) were unable to detect stomatal opening in response with light and CO_2 free air, using albino barley leaves which lacked detectable guard cell chlorophyll, and concluded that, guard cell chlorophyll is necessary for stomatal functioning. These findings plus the facts that stomatal chloroplasts contain chlorophylls 'a' and 'b' (Yemm and Willis, 1954), are capable of fixing $^{14}CO_2$ (Pearson and Milthorpe, 1974; Shaw and Maclachlan, 1954 a and b; Willmer et al., 1973) and are supposed to provide ATP necessary for active K^+

accumulation (Humble and Hsiao, 1969), have led many authors to conclude that the presence of chlorophyll in guard cells is necessary for stomatal operations and is universal for all functional stomata (Zucker, 1963).

This thesis indicates that the presence of guard cell chlorophyll in functioning stomata is not universal. Other findings of the thesis suggest that in *Paphiopedilum spps.* a unique stomatal mechanism may be in operation.

LITERATURE REVIEW

A. Introduction

The leaf epidermis functions as a barrier to gas exchange between the underlying mesophyll and the environment. Except for stomata, the continuous layer of thick walled, heavily cutinized epidermal cells acts as a relatively constant component of this resistance. Adjustments in stomatal pore aperture function as a variable resistance, controlling $C0_2$ influx to the photosynthetic apparatus and water loss to the environment. Stomatal researchers have not been able to fully explain how guard cells open and close. There is some agreement as to the basic mechanism, but at present, no one theory adequately accounts for all that is known about guard cell response to environmental factors.

The basis of all theories presented thus far, is that stomatal movements are the result of the development of a turgor differential between the guard cells and the subsidiary and/or neighboring epidermal cells (eg. Meidner and Mansfield, 1968). Stomata open when the guard cell turgor increases relative to that of the epidermal cells, and close when the reverse occurs. There is considerable evidence to suggest that in well watered plants, guard cell turgor increases are the result of decreases in guard cell solute potential (increases in solute concentration), followed by a passive influx of water from the epidermal cells (eg. Heath, 1959; Levitt, 1967). Increases in guard cell solute concentration can occur either by internal generation of solutes or by import of external solutes (Levitt, 1967). Internal generation of solutes has been attributed to hydrolysis of guard cell starch to sugars and/or organic acids (Levitt, 1967), and/or photosynthetic production of osmotically active substances (Levitt, 1974). The import of external solutes has been attributed mainly to influxes of inorganic ions, especially the monovalent cation - potassium (Hsiao, 1975). Analysis of changes in epidermal chemistry and inorganic ion concentrations suggests that both internal generation and import of solutes may be involved in stomatal operation (Levitt, 1974).

Recently, many advances have been made towards a more complete understanding of stomatal mechanisms. This literature review will discuss the aspects of epidermal anatomy which determine gas exchange and metabolic activity. It will also deal with inorganic ion flux and biochemical changes within the epidermis as related to stomatal movements. For more extensive reviews of stomatal physiology, the reader is referred to: Heath and Mansfield (1969), Hsiao (1975), Ketellaper (1963), Levitt (1967 and 1974), Meidner and Mansfield (1968), Pallas (1966), Raschke (1975), Stålfelt (1956) and Zelitch (1961, 1965 and 1969).

B. Epidermal Anatomy

As previously stated, the process of stomatal operation is dependent upon the development of turgor pressure differences between guard and other epidermal cells. The increase in guard cell turgor relative to epidermal cell turgor causes a deformation of the guard cells, resulting in an increase in pore aperture and increased gas exchange. The ability of guard cells to undergo these conformational changes is a function of: differently thickened cell walls (Schwendener, 1881; Von Mohl, 1856), the elastic modulus of the guard cell wall (DeMichelle and Sharpe, 1973), and radial micellation within guard cell walls (Voltz, 1952; Zeigenspeck, 1938 and 1955). The importance of radial micellation (the arrangement of cellulosic microfibrils radially out from the pore towards the dorsal cell wall), as discussed by Aylor, Parlange and Krikorian (1973), is that it prevents lateral guard cell expansion and causes the conformational change to be outward (i.e., increase in aperture). These conformational changes are enhanced because the guard cell ventral and upper tangential cell walls are free to move.

A layer of cuticle covers the guard, epidermal and substomatal chamber cell walls (Allaway and Setterfield, 1972). The layer of cuticle covering guard and epidermal cells is relatively thick, often extending to form a cuticular overlip above the stomatal pore (eg. Guttenberg, 1959). The chemical and physical nature of the cuticle is such that it is hydrophobic, the degree of which is known to vary with age (Holmgren, Jarvis and Jarvis, 1965), plant part, and species (Franke, 1967). The hydrophobic nature of the cuticle tends to decrease cellular water loss. Ectodesmata (interfibrillar spaces in cellulose cell walls, extending to, but not perforating the cuticular layer - Franke, 1967) have been shown using histochemical and audioradiographic techniques (Franke, 1967; Schnepf, 1959) to exist in a number of species. These techniques have shown a high ectodesmata density along the edges of the stomatal pores and along the dorsal anticlinal guard cell wall (Franke, 1964 a and b; Maercker, 1964, and 1965 a and b). However, they have not been well demonstrated by the electron microscope (Pallas and Mollenhauer, 1972b; Thomson and DeJournett, 1970). True pores in the cuticle have only been demonstrated in *Trifolium repens* and *Brassica oleracea* (Hall, 1967). Ectodesmata are thought to be involved in cutin deposition (Franke, 1967) and to increase water loss from the epidermis through the cuticle (peristomatal transpiration - Mearcker, 1965a).

The physiological significance of plasmodesmatal connections between guard and epidermal or subsidiary cells is unclear. The presence of plasmodesmata could allow symplastic assimilate and ion exchange between these cells by lowering the electrical resistance between the cells

(Robards, 1975), but may interfere with the development of a turgor differential (Hsiao, 1975). Most commonly, all epidermal cells are interconnected by plasmodesmata, however, guard to subsidiary or epidermal cell plasmodesmata are less frequent. Plasmodesmata between guard and epidermal cells are most commonly found in young tissues if at all, and not in mature tissues (Allaway and Setterfield, 1972; Kaufman et al., 1970; Singh and Srivastava, 1973; Srivastava and Singh, 1972; Thomson and DeJournett, 1970). However, Pallas and Mollenhauer (1972a), report the occurrence of plasmodesmata concentrated in pit-fields located in the cell wall between guard and epidermal cells of *Vicia* and *Nicotiana* Similarily, plasmodesmata between sister guard cells are present in younger stages (Brown and Johnson, 1962; Kaufman et al., 1970; Pallas and Mollenhauer, 1972a; Srivastava and Singh, 1972), but disappear with aging (Allaway and Setterfield, 1972; Thomson and DeJournett, 1970).

Ontogenetic studies (Kaufman et al., 1970; Landré, 1969; Pickett-Heaps and Northcote, 1966; Singh and Srivastava, 1973; Srivastava and Singh, 1972) have shown that young guard and other epidermal cells are similar prior to differentiation. These cells are densely cytoplasmic with thin cell walls and contain similar amounts of dictyosomes, spherosomes peroxisome like microbodies, endoplasmic reticulum, mitochondria, starch-less proplastids, and small vacuoles. At maturity, the other epidermal cells contain less ground cytoplasm (on a volume basis) than subsidiary (if present) or guard cells, and may contain large centrally located vacuoles with an alkaline pH (Srivastava and Singh, 1972). Epidermal cell plastids generally remain relatively undifferentiated and lack chlorophyll, grana, and starch (Pallas and Mollenhauer, 1972b; Pearson and Milthorpe, 1974; Singh and Srivastava, 1973; Srivastava and Singh, 1972). In *Nicotiana* (Pallas and Mollenhauer, 1972b), and in some ferns (Wylie, 1948), chlorophyll and starch have been reported in the epidermal plastids. When subsidiary cells are present they are more similar to epidermal cells than guard cells, and usually contain more plastids, mitochondria and a larger nucleus (Pearson and Milthorpe, 1974).

At maturity, normal guard cells may contain: numerous small vacuoles with an acidic pH (Singh and Srivastava, 1973), differentiated plastids, numerous mitochondria, and other cellular components such as dictyosomes, microbodies, spherosomes and endoplasmic reticulum. The guard cell plasmalemma may contain invaginations. The possible involvement of pinocytosis in stomatal operation has been suggested (Thomson and DeJournett, 1970), however, these invaginations have also been attributed to fixation and embedding problems (Pallas and Mollenhauer, 1972b). The occurrence of peroxisome like microbodies thought to contain the enzymes of glycolate metabolism (Tolbert et al., 1968), is somewhat variable. They are rarely found in the guard cells of *Vicia* and *Allium* (Allaway and Setterfield, 1972), but have been reported to be common in *Vicia* and *Nicotiana* (Pallas and Mollenhauer, 1972b), *Pisum* (Singh and Srivastava, 1973), and *Opuntia*(Thomson and DeJournett, 1970). The question of the involvement of glycolate metabolism in stomatal opening (Zelitch and Walker, 1964) remains undetermined based on these findings. Spherosomes, thought to serve as lipid storage and transport organelles (Pallas and Mollenhauer, 1972b) and to contain fluctuating concentrations of acid phosphatase (Sorokin and Sorokin, 1968), also vary in their occurrence. They have been reported to be uncommon in *Vicia* and *Allium* (Allaway and Setterfield, 1972) and to be abundant in the guard cells of *Campanula* (Sorokin and Sorokin, 1968). However, no correlation between the development of spherosomes and stomatal opening has been noted (Allaway and Setterfield, 1972). The endoplasmic reticulum tends to be well defined in young tissue and less well defined in mature tissue.

The number of guard cell mitochondria increases with age. They are more numerous per cross sectional area than in any other leaf cell. Allaway and Setterfield (1972) reported as many as 68 per guard cell profile in *Allium*. Mitochondria are 3 to 10 times more numerous than plastids and have well developed cristae. Although mitochondrial respiration is not believed to account for certain stomatal responses to light and CO_2, the high concentration of guard cell mitochondria indicates a possible significance in the production of organic acids and ATP (adenosine triphosphate).

The guard cell plastids increase in size, develop internal lamellae and grana stacks, and acquire starch grains as the cell matures (an exception being *Allium* which lacks starch - Allaway and Setterfield, 1972). The presence of guard cell chlorophyll is thought to be universal in functioning stomata (see Introduction p. 1). Typically there are 8-10 chloroplasts per guard cell, each somewhat smaller than mesophyll chloroplasts (Allaway and Setterfield, 1972; Pearson and Milthorpe, 1974). These plastids usually contain several grana (less than in mesophyll chloroplasts), with 4 thykaloids per granum (as many as 8 have been reported - Allaway and Setterfield, 1972). The plastids are fully capable of photosynthetically fixing $^{14}CO_2$ (Pearson and Milthorpe, 1974; Shaw and Maclachlan, 1954 a and b). Extensive peripheral reticulum (thought to be the site of PEP (phosphoenolpyruvate) carboxylase or to function to increase the effective surface area of the membrane - Laetsch, 1974) is characteristic of guard cell plastids. A possible role in stomatal operations has been suggested (Allaway and Setterfield, 1972).

In summary then, there is anatomical evidence to suggest that guard cell photosynthesis, starch hydrolysis and synthesis, and mitochondrial respiration may be intimately involved in guard cell mechanisms. Up to this time the relative importance of ectodesmata, plasmodesmata, pinocytosis, peroxisomes and spheresomes remains undetermined based on infrequent and varied evidence.

C. Epidermal Inorganic Ion Flux and Chemistry

Several studies have indicated a role for inorganic ion flux in stomatal operations. Iljin (1922) studied the effect of Group I elements on stomatal opening, and found that at high concentrations (greater than expected in the apoplast - 0.1 to 1 M) the order of effectiveness was $Li^+ > Na^+ > Cs^+ > K^+ > Rb^+$. Imamura (1943) found that at low concentrations the order of effectiveness was reversed, and that stainable concentrations of potassium occurred in the guard cells. Using epidermal strips, Fujino (1967) and Fischer (1971) found light activated K^+ stimulation of stomatal opening. All Group I elements tested (Li^+, Na^+, Rb^+, Cs^+) were found to cause increased stomatal opening with increased concentration in the external solution. But only K^+ and Rb^+ resulted in light and $C0_2$ free air activated increases in stomatal apertures (Humble and Hsiao, 1969). No specific anion was required to maintain the K^+ effect on guard cells in the epidermal strips of *Vicia faba* (Cl^-, Br^-, and $N03^-$ were tested as salts of K^+). The Group II elements Ca^{++} and Mg^{++} were found to be inhibitory to stomatal opening at moderate to low concentration levels (Humble and Hsiao, 1969; Iljin, 1922, Imamura, 1943; Willmer and

Mansfield, 1969).

In *Commelina* (Willmer and Mansfield, 1970), it was shown that treatment solutions of NaCl were as effective as KCl in causing stomatal opening. However, this work was criticized (Humble and Hsiao, 1970; Humble and Raschke, 1971), in that high concentrations were used (greater than 100 mM), and that no $CaCl_2$ was employed to preserve membrane integrity and ion selectivity (Pallaghy, 1970). Electron probe analysis has confirmed these criticisms, as no significant changes in guard cell Na^+ were detected when guard cells opened and closed (Humble and Raschke, 1971). Thus, Na^+ is assumed to play a minor role in stomatal operation.

Recent stomatal research has placed great emphasis on the role of potassium ions in stomatal movement. Willmer and Pallas (1973) and Dayanandan and Kaufman (1975) have shown that K^+ accumulates in the guard cells of a large number of species under opening conditions. Guard cell K^+ uptake has been estimated by $^{86}Rb^+$ (Fischer, 1971; Fischer and Hsiao, 1968; Humble and Hsiao, 1970; Mansfield and Jones, 1971), $^{42}K^+$ (Fischer, 1972; Pallaghy and Fischer, 1974), sodium cobaltinitrite stain for potassium (Allaway and Hsiao, 1973; Fischer, 1971 and 1972; Humble and Hsiao, 1970), electron proble analysis (Humble and Raschke, 1971; Raschke and Fellows, 1971; Sawhney and Zelitch, 1969), flame photometry (Allaway and Hsiao, 1973) and K^+

sensitive electrodes (Penny and Bowling, 1974). K^+ accumulation occurs in sufficient quantities so as to partially account for the noted changes in the guard cell osmotic potential if in association with an anion. The action spectra for K^+ accumulation in guard cells of isolated epidermal strips corresponded to that for stomatal opening in both isolated strips and leaf discs (Hsiao, Allaway and Evans, 1973). In *Commelina* (Penny and Bowling, 1974) and *Zea* (Pallaghy, 1971; Raschke and Fellows, 1971), the immediate source of potassium for guard cell accumulation is thought to be the subsidiary cells. Potassium leaves the subsidiary cells and accumulates in the guard cells as stomata open, and leaves the guard cells and accumulates in the subsidiary and epidermal cells as the stomata close. The origin of the potassium accumulated in the guard cells of *Vicia*, a species without subsidiary cells, is undetermined. As the epidermis takes up large amounts of exogenous K^+, it has been suggested that the mesophyll cells act as a source (Hsiao, 1975). However, various analyses have shown that there is considerable potassium in the epidermis (Allaway and Hsiao, 1973; Fischer, 1972) and that the amount in the epidermis is approximately the same in the open *vs.* closed stomata conditions (Willmer, Pallas and Jackson, 1974). K^+ fluxes have been shown to be active, based on Nernst analysis, in *Commelina* (Penny and Bowling, 1974), and are assumed to be so in other species (Hsiao, 1975).

The high concentration of K^+ accumulated in guard cells of open stomata requires electrochemical balancing (Humble and Raschke, 1971). This could occur either by concurrent uptake of inorganic and/or organic anions, internal generation of anions, or exchange of cations (Hsiao, 1975). The method of concurrent uptake of organic anions has been considered unlikely in the intact leaf, as stomata on epidermal strips still open when floating on solutions lacking organic anions. The import of inorganic anions, especially Cl^-, was assumed to play a minor role in stomatal operations of *Vicia* (Humble and Hsiao, 1969; Raschke and Humble, 1973). Electron probe analysis of *Vicia* stomatal complexes indicate that Cl^-, $PO_4^=$ and $SO_4^=$ are not transported in osmotically significant amounts as stomata open or close (Humble and Raschke, 1971). However, using $^{36}Cl^-$, Pallaghy and Fischer (1974) showed that 20% of the K^+ flux to guard cells of *Vicia* could be electrochemically balanced by Cl^- influx. In both *Commelina* and *Zea* species, a slightly different relationship occurs. In *Zea*, a Cl^- shuttle occurs similar to that for K^+ as stomata open and close, and approximately .4Cl^- are transported with every K^+ ion (Raschke and Fellows, 1971). In *Commelina*, Cl^- fluxes follow K^+ fluxes, however, the relative amounts are undetermined (Willmer and Pallas, 1974). Thus, the import of inorganic anions is involved in the electrochemical balancing of the potassium concentrations in guard cells of open stomata, while the relative importance varies from species to species.

Raschke and Humble (1973) showed a significant exchange of H^+ ions for K^+ as stomata of *Vicia* opened, and suggested that these H^+ ions were the product of organic anion production in the guard cells. In *Vicia* (Allaway, 1973) and *Commelina* (Pearson, 1973 and 1975; Pearson and Milthorpe, 1974), it was shown that the organic acid, malate, concentration in guard cells increased in osmotically significant amounts as the stomata opened. In *Vicia*, the change in epidermal malate could have balanced approximately half of the K^+ which was accumulated during stomatal opening (Allaway, 1973). Concentration changes in other organic acids, glyceric and citric acids, have been measured in the epidermis of *Vicia* as stomata open (Pallas and Wright, 1973). Similarly increases in guard cell malate and aspartate have been measured as stomata open in *Commelina* and *Tulipa* species (Willmer and Dittrich, 1974). Thus, organic acid production in guard cells of opening stomata is thought to assist in the electrochemical balancing of K^+ by generating anions (for potassium salt formation) and H^+ ions for exchange. Malate production from non-osmotically active substances such as starch could also account for part of the observed osmotic potential changes in guard cells as they open. The origin of guard cell malate is not yet known; some possibilities are: starch degradation (Allaway, 1973) - starch content in guard cells decreases with stomatal opening (Mouravieff, 1974); CO_2 fixation by PEP carboxylase - this enzyme has been shown to occur in high concentrations in the epidermis of *Tulipa* and *Commelina* (Willmer et al., 1973); or amino acids entering the TCA cycle - ^{14}C amino acids decline as ^{14}C organic acids increase in the epidermis of *Commelina* during opening (Pearson and Milthorpe, 1974).

The pathotoxin from *Helminthosporum maydis* (Arntzen, Haugh, and Bobick, 1973), the tentoxin of *Alternaria tenuis* (Durbin, Uchytil, and Sparapano, 1973), and the hormone abscisic acid (Horton and Moran, 1972; Mansfield and Jones, 1971) all prevent stomatal opening by preventing K^+ accumulation in guard cells. The fungal toxin Fusicoccin has been shown to induce wilting by stimulating stomatal opening and K^+ accumulation in guard cells (Squire and Mansfield, 1974; Turner 1973).

Thus, the involvement of K^+ in stomatal functioning is now well established (Hsiao, 1975). Heretofore, no exception was known in which K^+ is not at least one of the major osmotica in stomatal operation. The involvement of organic acids, especially malate as an electrochemical balancer for K^+ uptake, has also been detected in a number of species. These findings have recently been incorporated into theories presented by Hsiao (1975), Levitt (1974), and Raschke (1975).

Although many advances have been made towards a more complete understanding of stomatal physiology, a weakness may exist. Except in a few cases - a survey of potassium distribution in the leaf epidermis (Dayanandan and Kaufman, 1975; Willmer and Pallas, 1973) and epidermal morphology (several publications by Inamadar, eg. Inamadar, Bhatt and Patel, 1973) in a variety of species - studies were limited to a few species. Proposed stomatal mechanisms as understood based on this limited survey may be misleading in terms of a universal stomatal mechanism.

PART 1 - ANATOMICAL INVESTIGATIONS

A. Introduction

Functional guard cells are known to occur on epidermal surfaces of all angiosperms and gymnosperms except some aquatics, and some cycads, horsetails, ferns, mosses and liverworts (Zucker, 1963). The presence of chlorophyll in functional guard cells is thought to be universal (Zucker, 1963), and there is considerable evidence to suggest that the presence of chlorophyll is necessary in order that the guard cells are functional (p. 1). However, it was observed by Dr. James Mayo (Dept. of Botany, University of Alberta, Edmonton, Alberta, Canada), that the guard cells of some lady's slipper orchids, *Paphiopedilum spp.*, lacked green coloration and therefore, chlorophyll. Light and electron microscopy studies were undertaken to ascertain the structural and chemical nature of the guard and epidermal cells of these species.

B. Materials and Methods

1. Light microscopy

Unless otherwise stated, the *Paphiopedilum* species used in this thesis originated from Carolina Orchids Inc. (USA), and were kept in the University of Alberta Botany Greenhouses. For purposes of comparison, other *Paphiopedilum spp.* were obtained from the suppliers listed in

Appendix A.

The lady's slipper orchids, *Paphiopedilum fairrieanum, P. harissanium, P. insigne,* and *P. leeanum,* were used in conjunction with *Zebrina pendula* in the microscopic studies. *Z. pendula* was employed as the control, as it is known to contain normal chlorophyllous guard cell plastids (Alvim, 1949).

Light microscopic studies were carried out with a Zeiss photomicroscope. Fresh abaxial and adaxial epidermal peels were briefly washed and mounted in distilled water for microscopic observation. Various age classes of leaves were observed, and photographs were taken within 30 min of peeling.

The presence or absence of chlorophyll was determined by the technique of fluorescence microscopy. Transmitted light (420 nm), from a 200W/4 super pressure mercury vapor lamp, was used to excite chlorophyll molecules. A barrier filter insert (Zeiss 47 25 47) was employed to block all wavelengths except those emitted by excited chlorophyll (668 nm, eg. Nobel, 1974). The exciter and barrier filter settings were those suggested in the Zeiss Fluorescence Microscope Manual.

Epidermal strips were also stained with a lipid specific stain (Sudan IV), and a starch stain (potassium iodide). Thick plastic sections (.5 - 2 um) of material prepared for electron microscopy were used to make measurements of cell dimensions. The sections were stained with periodic acid/Schiff's (PAS - stains carbohydrates with vicinal hydroxyl groups, such as, starch, hemicelluloses and pectins), toluidine blue (TB - cationic dye staining carboxylated polysaccharides and macromolecules with free phosphate groups), ruthenium red (RR-stains pectins), and aniline blue black (ABB - stains proteins).

2. Electron microscopy

Mature leaves of *P. leeanum* which had been in the light for at least 2 hours prior to sampling were used in the electron microscopy study. Leaf segments (ca. 2X2X3 mm) were dissected and immediately fixed with 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7) for 24 hours at 10 °C. The tissue was washed with phosphate buffer (pH 7) and post-fixed with phosphate buffered 2% osmium tetroxide for 4 hours at room temperature. It was then washed and dehydrated in an ethanol, propylene-oxide series; infiltrated with aralidite for 24 hours, and embedded at 60 °C. Some difficulty was experienced with sectioning, and as a result, epoxy resin infiltration and embedding was employed. (Spurr, 1969). Glass knives were made on a LKB Knifemaker type 7801A and were then used to cut thin and thick sections on a Sorvall Porter-Blum MT-2 microtome. Sections were mounted on 100 and 200 mesh copper grids and stained with 2% uranyl acetate for 2 hours, followed by 0.2% lead citrate for 2 min. Micrographs were taken on Phillips TEM 200 and 300 electron microscopes.

C. Results

1. Stomatal anatomy

The adaxial leaf surface of all the *Paphiopedilum spp.* that were studied lacked guard cells. The abaxial surface of these species contained guard cells and epidermal cells (Fig. 1). The guard cells were elliptically shaped, and no epidermal cells were distinguishable as subsidiary cells. The average number of guard and epidermal cells per mm^2 of leaf surface was found to be 51.9 and 164, respectively (Table 1). The average external guard cell dimensions of *P. leeanum* were 71.2 um long and 51.9 um wide. For volume and dimension measurements, the internal structure of a guard cell was visualized as being composed of a dorsally located cylinder and a ventrally located rectangle (Table 1 and Fig. 2). The width of the rectangular portion was found to approximate one-half the diameter of the cylinder. The stomatal index (Meidner and Mansfield, 1968, p. 6) was 17.6%.

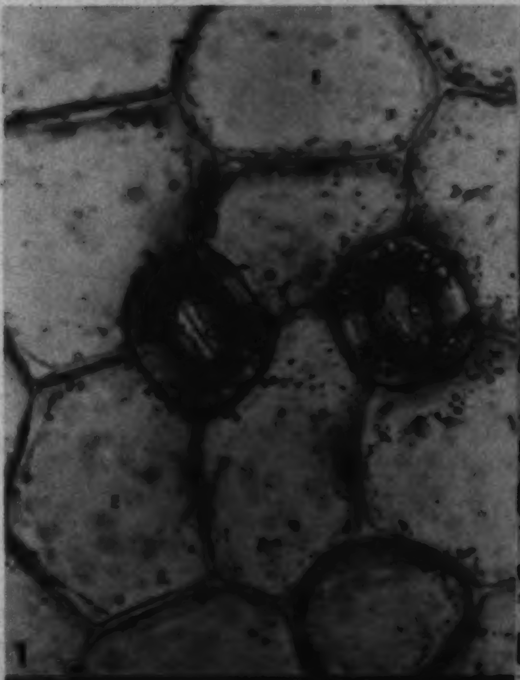
The upper and lower tangential guard cell walls of *P. leeanum* were thick, while the anticlinal walls were relatively thin. All walls, except the dorsal anticlinal, were noticeably covered by a layer of cuticle. The upper tangential wall was covered by a very extensive cuticular overlip which formed a supra-stomatal chamber (Fig. 2).

Figure 1. Bright field micrograph, abaxial leaf surface of *Paphiopedilum leeanum*. Live tissue after epidermal peeling process, X 320.

Figure 2. Electron micrograph, guard cells of *P. leeanum* Shows the thick tangential cell walls (CW) of the guard cells, large cuticular overlip (cl), suprastomatal chamber (ssc), neighboring epidermal cell (E), large central vacoule (V), osmiophillic plastids (P), starch grains (S), and invagination of epidermal cell plasmalemma (I). The internal volume of a guard cell can be seen as a combination of a ventrally (closest to pore) located rectangle and a dorsally located cylinder (the width of the rectangle approximately equal to the diameter of the cylinder). The dark string like structures are wrinkles in the section. X 2450.

Figure 3. Fluorescent micrograph, guard cells of *Zebrina pendula*. The guard cell chloroplasts appear as white bodies, the faint white areas in the background are due to mesophyllic chlorophyll. The white bodies as seen in this photograph fluoresced a distinct red (a red sensitive film was used to take the photograph). Exciting wavelength of 420 nm and barrier filter insert Zeiss 47 25 47 were used in conjunction with a Zeiss photomicroscope. X 806.

Figure 4. Fluorescent micrograph, guard cells of *Z. pendula* showing cell structure using a non red sensitive film. The two guard cells with their fluorescent chloroplasts, subsidiary cells (SC) and the fluorescent underlying mesophyll chloroplasts can be seen. The subsidiary cell nucleus (N) is also prevalent. Exciting wavelength of 420 nm and barrier filter insert Zeiss 47 25 47 were used in conjunction with a Zeiss photomicroscope. X 806.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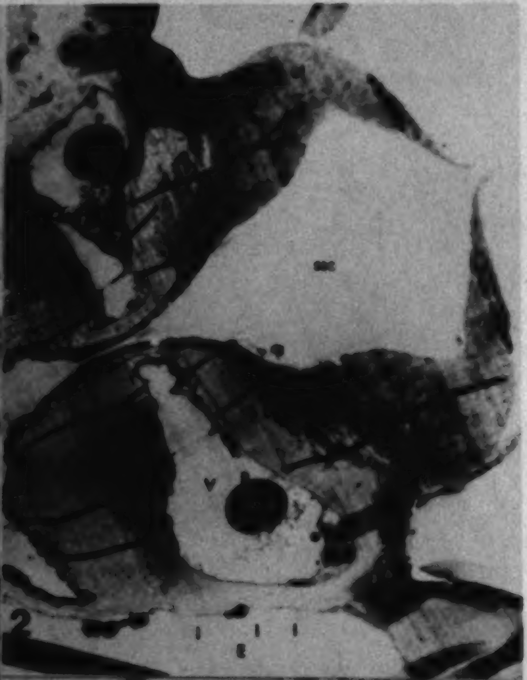
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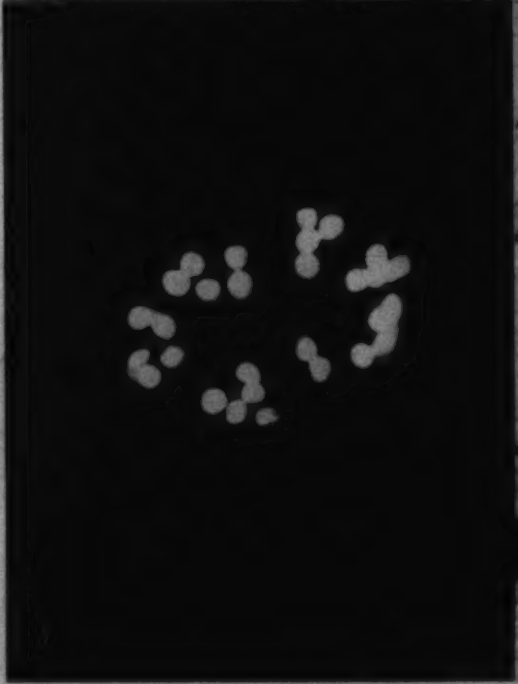

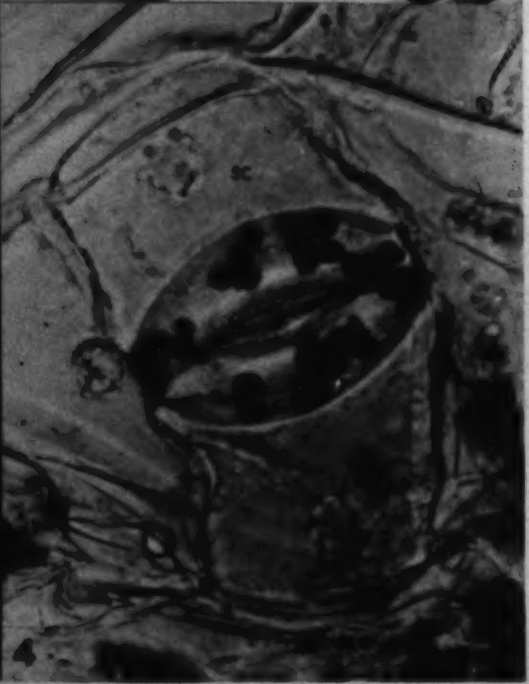
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TABLE 1 - Dimensions and number of cell types, in the epidermis (abaxial) for leaves of *P. leeanum*. Measurements from fixed and live material. The internal structure of a guard cell was visualized as being composed of a dorsally located cylinder and a ventrally located rectangle.

	Guard Cell Frequency	Epidermal Cell Frequency	External Stomatal Dimensions		Internal Guard Cell Dimensions			Internal epidermal Cell Dimensions	
			length	width	length	width[1]	depth[2]	length width	depth
Ave. of all readings	$51.9/mm^2$	$164/mm^2$	71.2um	51.9um	67.5um	14.3um	1.71um	75.3um	68um

(1) diameter of cylindrical portion

(2) from upper and lower tangential cell walls in the rectangular portion of the guard cell.

This cuticular overlip made the direct measurement of stomatal pore aperture difficult. Outbranching of cell wall material into this overlip was noted (Fig. 2). No ectodesmata or plasmodesmata (between sister guard cells or between guard and epidermal cells) were seen.

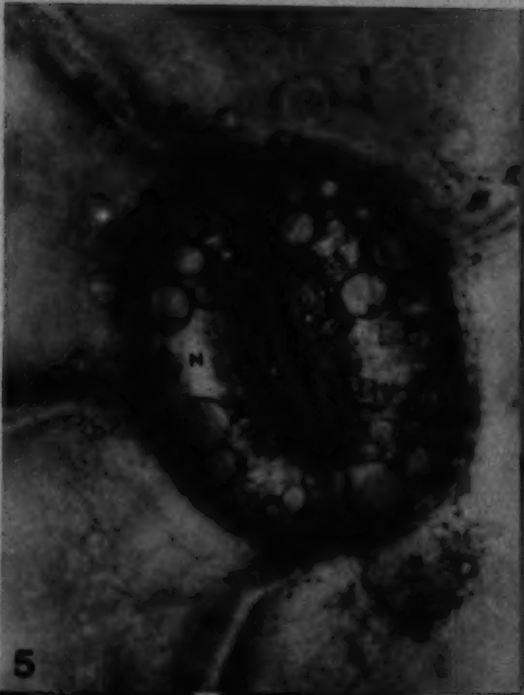
The guard and epidermal cells of *Paphiopedilum spp.* contained colorless plastids, which were distinctly different from the green mesophyll chloroplasts of *Paphiopedilum* and the guard cell chloroplasts of *Z. pendula.* The mesophyll plastids of *Paphiopedilum spp.* and *Z. pendula,* and the guard cell plastids of *Z. pendula,* fluoresced a distinct red under fluorescence microscopy. A black and white print of a color slide (Kodak PCF 135) is shown in Fig. 3 (red sensitive film). A similar picture of *P. leeanum* could not be taken, as there was no plastid or cytoplasmic fluorescence in either the guard or the epidermal cells (i.e. the picture was black). In order to more clearly demonstrate the lack of fluorescence, the barrier filter settings were changed so that the chloroplasts of *Z. pendula* emitted a strong red fluroescence and the cellular structure could be photographed as well. *Z. pendula* (Fig. 4) and *P. leeanum* (Fig. 5) were photographed under these conditions using a non-red sensitive film (Kodak, Panatomic X). The red fluorescence of *Z. pendula* guard cell chloroplasts appear as dark bodies, whereas, the non-fluorescing guard cell plastids and cytoplasm of *P. leeanum* appear

Figure 5. Fluorescent micrograph, guard cells of *P. leeanum* showing cell structure using a non red sensitive film. The guard cell and epidermal cell nucleus (N) are evident. No guard cell plastid (P) or cytoplasmic fluorescence is detectable. Slight shading is due to diffraction by membranes, cell wall or cuticle. Starch grain (S) can be seen. Exciting wavelength of 420 nm, barrier filter insert Zeiss 47 25 47 were used in conjunction with a Zeiss photomicroscope. X 806.

Figure 6. Bright field micrograph, thick section of *P. leeanum* guard cells stained with periodic acid/Schiff's (PAS). Shows the intercellular air space (ias) below the guard cells, and the suprastomatal chamber (ssc). The guard cell plastid (P) did not noticeably stain with PAS, the dark color is due to staining with osmium tetroxide. The other guard cell bodies produced a strong red color with PAS, and were interpreted as starch grains (S). Cell walls were stained red. X 704

Figure 7. Electron micrograph, guard cell leucoplasts of *P. leeanum*. The starch grains (S) were membrane bound, but no appressed thykaloids were observed. Photograph taken on a Phillips EM 300. X 23,100.

Figure 8. Electron micrograph, guard cell mitochondria (M) of *P. leeanum*. The mitochondria are numerous and appear well developed based on the internal cristae structure. The cell wall (CW) contains noticeable micellation. Photograph taken on a Phillips EM 200. X 29,750.



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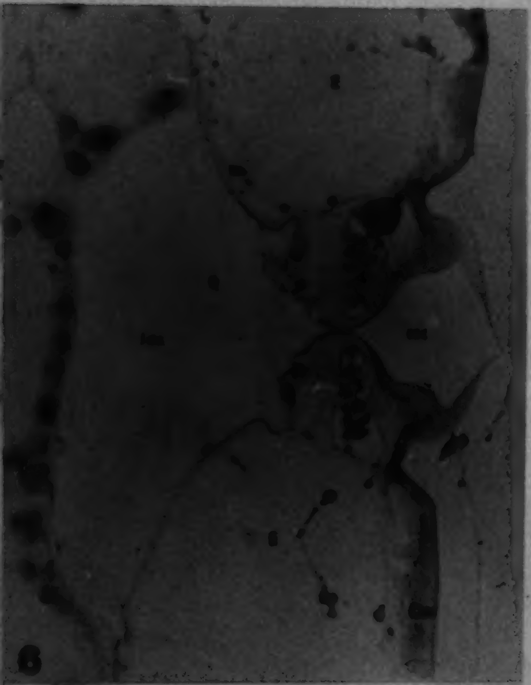

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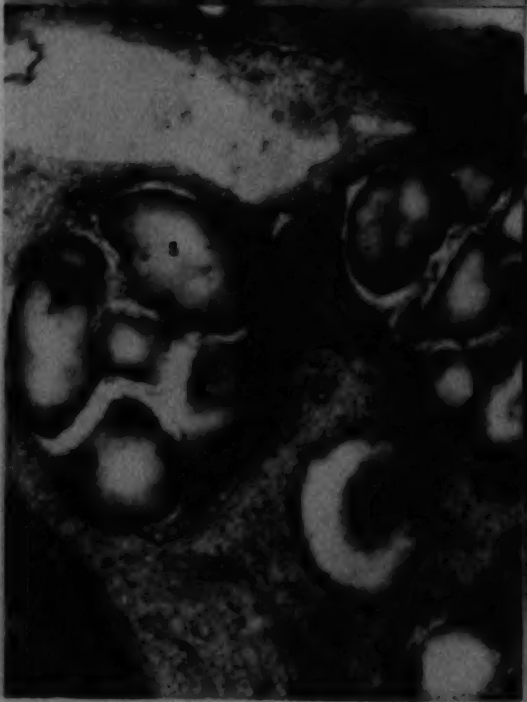

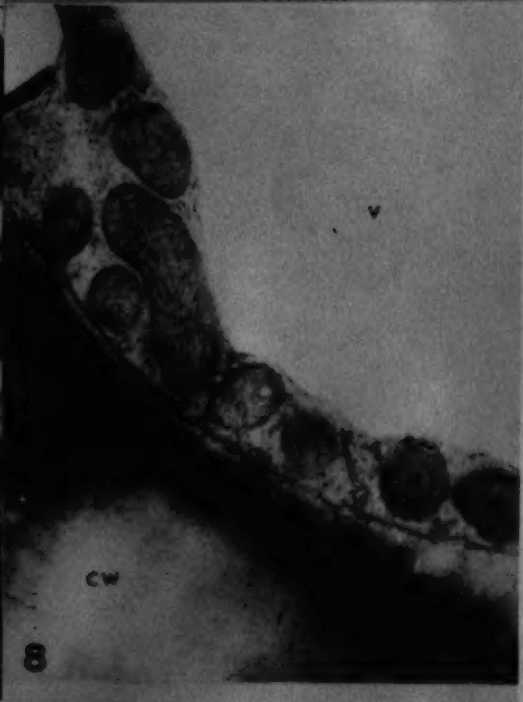

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clear, indicating a lack of chlorophyll. The mesophyll chloroplasts of *P. leeanum* also fluoresced red under these settings. Thus, the guard and epidermal cells of *P. leeanum* and all other *Paphiopedilum spp.* examined, did not contain detectable levels of chlorophyll. However, the mesophyll of these species contained 'normal' chlorophyllous plastids.

The guard cells of *P. leeanum* contained differently shaped and sized plastids (Fig. 5). The large spherical plastids showed a positive reaction with Sudan IV. They also stained heavily with osmium tetroxide (Fig. 2), thus suggesting a high level of unsaturated lipids. The size of these plastids varied, and occassionally approximating the size of the plastids in neighboring epidermal cells (which also stained with Sudan IV). The smaller more irregular guard cell plastids reacted intensely with PAS to produce a dark red color (Fig. 6). They were observed at the electron microscope level to be starch containing plastids (Figs. 2 and 7). In no case were any chlorophyll containing membranes (appressed thykaloids) observed in any guard cell plastids. Numerous well developed mitochondria were observed in the guard cells (Fig. 8). They outnumbered any other organelle, and as many as 27 were counted in a single thin section profile of a guard cell. The guard cells also contained large undissected vacuoles, a nucleus and infrequent peroxisome, microbody and spherosome like structures.

2. Epidermal anatomy

The uniseriate layer of adaxial and abaxial epidermal cells of *P. leeanum* were observed to be different in size, but relatively similar in structure and content. Both were highly vacuolate, with a thin layer of cytoplasm lining the plasmalemma (Fig. 9). The thick outer tangential cell walls contained considerable lamellation (Fig. 10) and stained red with ruthenium red. There was heavy ruthenium red staining at the junction of the outer tangential and anticlinal cell walls. The anticlinal cell walls of the adaxial surface epidermal cells were undulating, whereas, those of the abaxial surface were straight (Fig. 9). Several plasmodesmatal pit fields were noted between epidermal cells (Fig. 11). Epidermal cells were similarly connected to mesophyll cells (Fig. 12).

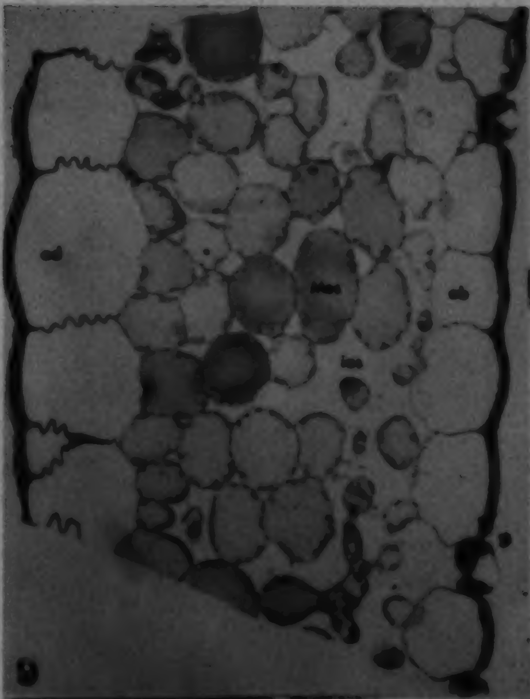
The cytoplasmic content of epidermal cells consisted mainly of a nucleus and several plastids (Fig. 13). At no time were any leucoplasts or chloroplasts observed in these cells. The epidermal plastids were membrane bound, usually, but not always, containing osmiophillic bodies (Fig. 13). The osmiophilic bodies did not appear to be membrane bound, but occasionally were associated with a prolamellar body (Fig. 11). Sudan IV and osmium tetroxide staining suggested that these bodies contained large quantities of unsaturated lipids, as in the guard

Figure 9. Bright field micrograph, thick section of the leaf of *P. leeanum*, stained with toluidine blue (TB). The abaxial (ab) and adaxial (ad) epidermis are shown. In the adaxial epidermis the anticlinal cell walls undulate whereas those of the abaxial epidermis do not. All cell walls take up the stain especially the outer tangential walls of epidermal cells, and guard cell walls. X 144.

Figure 10. Electron micrograph, thick section of *P. leeanum*, upper tangential epidermal cell wall. Note the difference in size between the tangential and anticlinal cell walls. The cuticle (cut) is seen to be composed of several layers. Considerable lamellation is seen in the epidermal cell walls mag. was X.

Figure 11. Electron micrograph, pit field (PF) between two abaxial epidermal cells of *P. leeanum*. A number of individual plasmodesmata (PL) are seen in the area where the cell is constricted. A poorly preserved mitochondria is also seen. X 42,560.

Figure 12. Electron micrograph, pit field (PF) between a mesophyll (MES) and an abaxial epidermal (E) cell of *P. leeanum*. In this case the section was over stained, however, the pit field (PF) can be seen. Characteristic epidermal plastids (P) and mesophyll chloroplast (C) can be seen. X 8740.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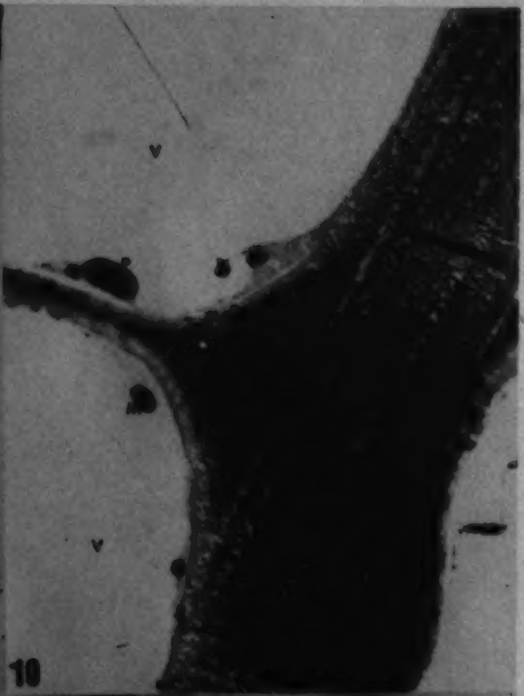
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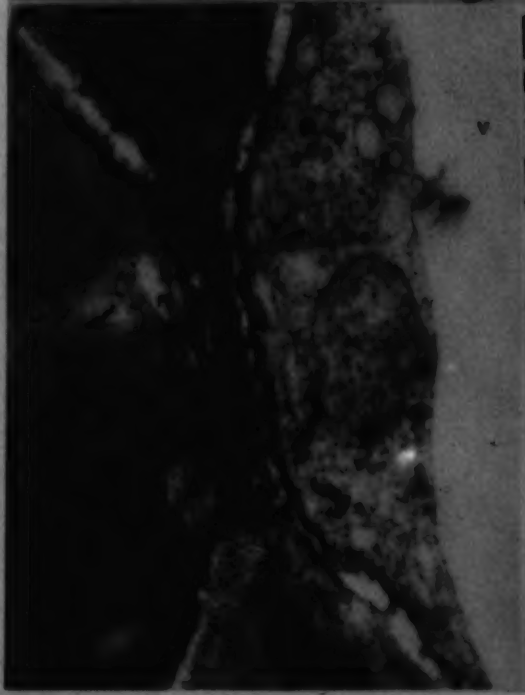
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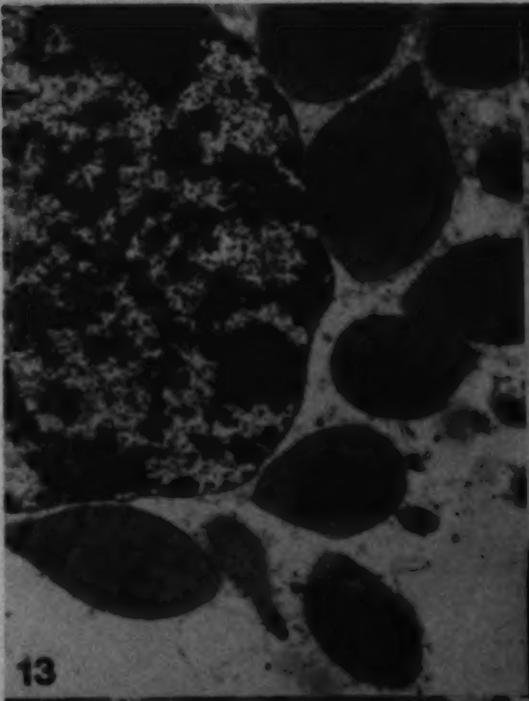
Figure 13. Electron micrograph, abaxial epidermal cell of *P. leeanum*. Plastids (P) with and without osmiophillic inclusions are shown. X6468.

Figure 14. Electron micrograph, abaxial leaf epidermal cell plastid of *P. leeanum*. The etio-elaioplast (Et-El) is a high magnification micrograph of Fig. 13. Note the prominent prolamellar body (Pb) and dark osmiophillic central portion of the plastid. A mitochondrion (M) can be seen as well X 23,760.

Figure 15. Electron micrograph, mesophyll cell of *P. leeanum*. The two chloroplasts (C) contain grana (g), and saturated lipid inclusions (sl). The mesophyll nucleus (N) and mitochondria (M) are also seen. This cell represents a mesophyll cell in which large starch inclusions in the chloroplasts are missing. X 21,600.

Figure 16. Electron micrograph, mesophyll chloroplast of *P. leeanum*. Note the extensive grana stacks (g), small osmiophillic inclusions. X 11200.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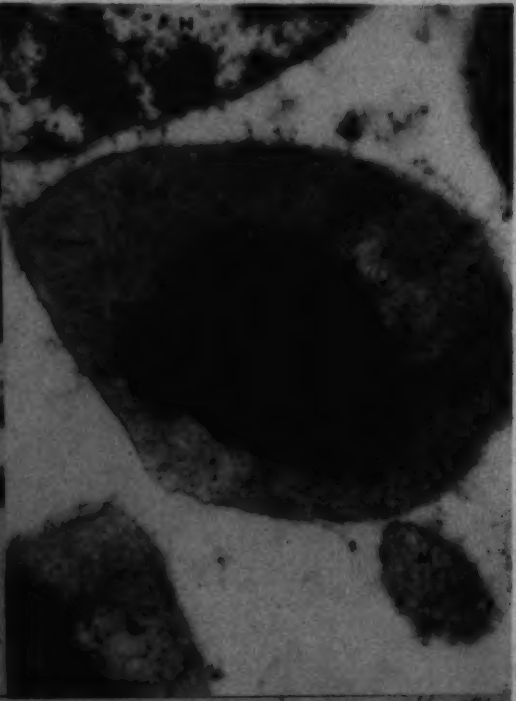
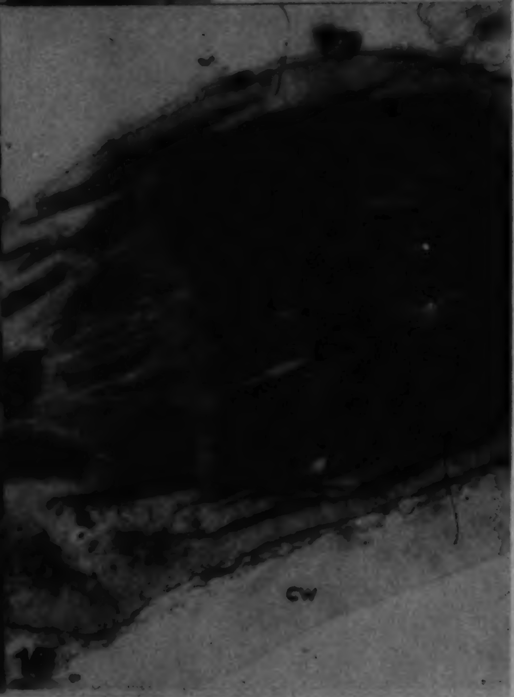




cell plastids. Occasional, poorly developed mitochondria, endoplasmic reticulum (figs. 11 and 14), and plasmalemma invaginations adjacent to guard cells (Fig. 2) were observed.

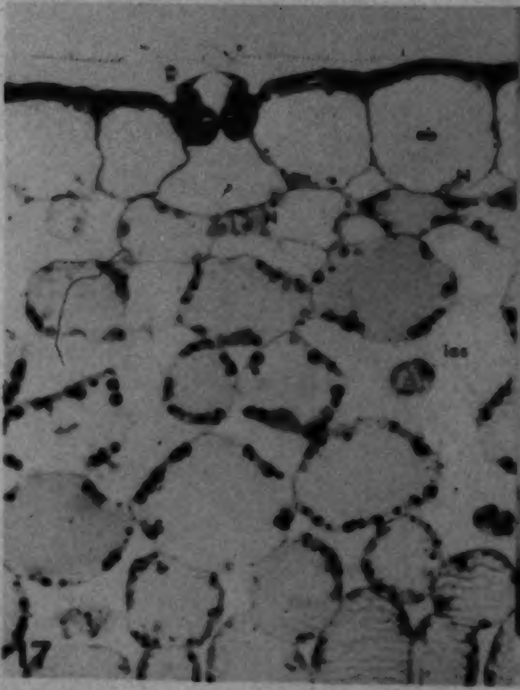
3. Mesophyll anatomy

Generally, the mesophyll cytoplasm per cell profile area was greater than the epidermal cells, but less than guard cells. The layer of spongy mesophyll was 1 to 2 cells thick, while the remainder of the mesophyll was defined as palisade (Fig. 9). The mesophyll cell walls were relatively thin and the intercellular air spaces were conspicuous.

The mesophyll cells contained mainly chloroplasts, few mitochondria, a nucleus, and occasional microbodies and endoplasmic reticulum (Fig. 15). The green, chlorophyll containing mesophyll chloroplasts were of two kinds. The first layer of both the spongy and the palisade cells contained chloroplasts with thykaloids stacked to form grana, stroma lamella, stroma, lipid inclusions (saturated), and small osmiophillic bodies (Figs. 15 and 16). The chloroplasts in the remaining mesophyll contained similar structures plus large spherical bodies. These bodies were not osmiophillic but showed a strong reaction with PAS (Fig. 17) - similar in intensity to that of the guard cell leucoplasts (Fig. 6). Electron microscopy (Fig. 18), indicated that these bodies were large starch grains. The

Figure 17. Bright field micrograph, thick section of the leaf of *P. leeanum* stained with periodic acid Schiff's (PAS) and toluidine blue (TB). The following color reactions were noted: all cell walls were blue, the guard and upper tangential epidermal cell walls were dark blue with noticeable lamellation, guard cell leucoplasts and starch inclusions of mesophyll chloroplasts (S) were dark blue, abaxial epidermal and guard cell elaioplasts as well as the saturated lipid inclusions of mesophyll chloroplasts (sl) and the cuticle did not noticeably stain but are dark as a result of osmium tetroxide staining. The section shows a lack of starch inclusions (S) in the mesophyll chloroplasts of cells adjacent to the abaxial epidermis (ab). The starch inclusions were common in the remaining mesophyll cells. X 250.

Figure 18. Electron micrograph, *P. leeanum*, mesophyll chloroplast containing large starch body (S). This chloroplast is representative of chloroplasts in mesophyll cells other than those immediately adjacent to the epidermal cells. Saturated lipid inclusions (sl) and other chloroplast components are shown, as well as neighboring mitochondria (M) and the mesophyll cell wall (CW). X 14,400.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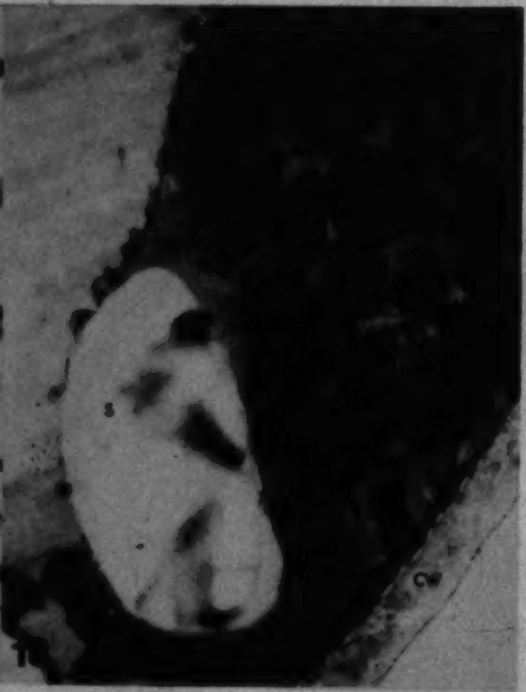


stacking of thykaloids to form grana was occasionally quite extensive (Fig. 16).

D. Discussion

The hypostomatous leaf surfaces of the lady's slipper orchid, *P. leeanum*, contain 51.9 elliptically shaped guard cells and 164 epidermal cells per mm^2. This stomatal frequency is low; Eckerson (1908) reported 200 guard cells per mm^2 as average for common greenhouse plants. Meidner and Mansfield (1968) noted that decreasing stomatal frequencies were correlated with increasing guard cell dimensions. The measurements of *P. leeanum* stomata support this observation in that the average stomatal length (71.2 u) and width (51.9 u) are considerably larger than average (for example, Meidner and Mansfield, 1968 quote the following lengths and widths: *Allium cepa*, 42 u long and 38 u wide; *Nicotiana tabacum*, 31 u long and 25 u wide; *Vicia faba*, 46 u long and 25 u wide).

The outer tangential cell walls of adaxial and abaxial epidermal cells, and both tangential guard cell walls are thick. Differentially thickened guard cell walls and radial micellation are thought to play a role in stomatal movements (Aylor et al., 1973). Both were noted in guard cells of *P. leeanum*, however, no further study was made of their relative importance. Ruthenium red staining indicates that the extensive lamellation of cell wall material (especially in the region where the tangential and anticlinal epidermal cell walls meet) may be due to cellulose rich, pectin poor - celluose poor, pectin rich layering.

No plasmodesmatal connections were observed between sister guard cells or guard and epidermal cells. This would allow for the development of a turgor differential between the guard and epidermal cells, but could increase the resistance to organic or inorganic molecule exchange. However, the common wall between these cells is relatively thin, and its resistance characteristics are unknown. The significance of the occasional invaginations of the epidermal cell plasmalemma (Fig. 2) is undetermined. Numerous plasmodesmatal connections between the epidermal cells suggest that the epidermal cells may act as a single osmotic unit in terms of stomatal operation. Thus the intercellular exchange of molecules within the epidermal cells may experience relatively small resistances. The plasmodesmata occurred in pit fields (ca. 8-10 per pit field), and contained an apparently osmiophillic core. There are epidermal to mesophyll plasmodesmata (again in pit fields). The frequency of occurrence is unknown. However, the possibility for some level of mesophyllic control of stomatal movements and/or metabolism via the epidermal cells exists.

All cell walls exposed to air are covered with a cuticular layer presumed to offer resistance to water loss. The cuticle is especially obvious on the epidermal and guard cells. The very extensive cuticular overlip (Fig. 2) above the guard cells forms a suprastomatal chamber which would affect the relative stomatal pore aperture and increase the effective pore length (both are known to increase the resistance to water vapor diffusion). The structural and chemical nature of the cuticle is unknown; both these factors are known to be important in determining the resistance to water flow that the cuticle offers (Kamp, 1930). No ectodesmata were present in the material observed, however, the histochemical and audioradiographic methods of Maercker (1964 and 1965 a and b) and Schnepf (1959) were not employed. Occasional outbranching of guard cell wall material into the cuticular overlip was noted (Fig. 2). However, these were not interpreted as ectodesmata.

The order or cytoplasmic content on a volume basis in the leaf cells of *P. leeanum* is guard > mesophyll > abaxial > adaxial epidermal cells. In terms of cytoplasmic differences between the cells, the most striking is the numbers of mitochondria and the types of plastids. Guard cells contain numerous well developed mitochondria (the extent of development determined by internal cristae formation). Allaway and Setterfield (1972) and Pearson and Milthorpe (1974) observed a similar situation for *Vicia* and *Commelina*. The number and development of guard cell mitochondria suggest a very high capacity for organic acid and ATP production. Both have been implicated in stomatal mechanisms (eg. Levitt, 1974).

The guard and epidermal cells of *P. leeanum* do not contain detectable levels of chlorophyll, whereas the mesophyll cells contain considerable amounts. Guard cells contain two major types of plastids, leucoplasts and elaioplasts; epidermal cells contain elaio and/or etioplasts; and the mesophyll cells contain chloroplasts of two types, those with starch inclusions and those without.

Some interesting questions are raised as to the origin of the guard cell leucoplasts and elaioplasts. Badenhuizen (1966) suggested that in the guard cells of coffee, the chloroplasts degenerated to form colorless leucoplasts. The formation of starch granules was seen to occur between grana, and increased in size to occupy most of the plastid stroma. The guard cell starch was assumed to be initially formed from local photosynthetically produced sugars (assimilatory starch), and then to complete its development aided by imported sugars (reserve starch). As the guard cells of *P. leeanum* do not contain any chlorophyll, or structures resembling grana, it can be assumed that the leucoplasts contain reserve starch. As the epidermal cells are also chlorophyll free, it is assumed that the origin of the guard cell starch is the mesophyll chloroplasts. The presence of plasmodesmatal connections from the mesophyll cells to the epidermal cells and throughout the epidermal cells, would facilitate the ready transfer of metabolites to the epidermal-guard cell wall. Badenhuizen (1969) states that it is generally found that all plastids have the ability to form starch if supplied with a sufficient quantity of sugars. Winkler (1898 - quoted in Badenhuizen, 1969) noted an exception to this in the epidermal plastids of 'orchids'. This observation could account for the lack of starch in the epidermal cells of *P. leeanum*. The epidermal plastids may lack the necessary enzymes (phosphorylase has been shown to be lacking in epidermal cells whereas it is present in guard cells - Yin and Tung, 1948) or may maintain an unsuitable pH. Srivastava and Singh (1972) have suggested that the pH of *Zea mays* epidermal cells is basic, a condition not favorable for starch synthesis. The guard cells of *P. leeanum* may contain the necessary enzymes and/or like other guard cells may maintain at various times a favorable pH for starch formation (Pallas, 1966).

Badenhuizen (1966) also noted that the leucoplasts of coffee guard cells experienced a further change in that the starch broke down and was later replaced by oil bodies (osmiophillic). Thus, the elaioplasts of *P. leeanum* guard cells may have been formed from the breakdown or conversion of leucoplasts. No real evidence for this sequential development was observed in *P. leeanum*. The guard cell elaioplasts resemble the etio-elaioplasts of the epidermal cells in that they contain a high concentration of unsaturated lipids. However, the sequence of development for the epidermal plastids may be quite different from that suggested above for guard cell elaioplasts. The epidermal plastids resemble a type of proplastid, an etioplast. The etioplast is a proplastid which has been kept in the dark, and is characterized by its prolamellar body. Upon illumination, the prolamellar body dissociates and its membranes form the thykaloids, which eventually form the chlorophyll containing grana (Rhodes and Yemm, 1966; Robertson and Laetsch, 1974). Thus, in the case of the epidermal cells of *P. leeanum*, it appears as if the epidermal plastids have experienced an arrested development, the cause of which is unknown. The epidermal plastids are considerably different from the etioplasts reported for other species in that they contain large concentrations of osmiophillic bodies (unsaturated lipids). Most etioplasts contain only an occasional small osmiophillic body. Although no electron microscopic evidence is available to establish the presence of prolamellar bodies in the guard cell elaioplasts of *P. leeanum*, the similar staining characteristics (Sudan IV and osmium tetroxide) suggest that the guard cell elaioplasts may have a similar origin, as those of the epidermal cells.

The mesophyll plastids are typical chloroplasts with well developed stroma lamellae, grana, lipid inclusions, small osmiophillic bodies and no peripheral reticulum. Peripheral reticulum is more characteristic of C4 chloroplasts (Laetsch, 1974). The first layer of spongy and palisade cells did not contain any large starch bodies, which are consistently found in the remaining chloroplasts (first layer being synonymous with the layer of mesophyll cells which make direct contact with the epidermal cells). Laetsch (1974) states that any chloroplast can form starch, provided that, its internal sugar concentration is allowed to build up (i.e. it is separated from its sink or its supply is increased from external sources). This is especially noticeable in C4 mesophyll chloroplasts which will form starch if separated from their sink (bundle sheath cells) or treated with external sucrose (Laetsch, 1974). Starch is normally not found in these cells. The epidermal cells of *P. leeanum* which do not contain any photosynthetic apparatus must obtain their supply of metabolites from the mesophyll cells. Possibly the guard and epidermal cells act as a 'sink', which depletes the first layer of mesophyll cell sugar levels to such a degree that starch bodies do not form. Plasmodesmatal connections between mesophyll cells were not observed, a situation which would allow for localized depletion of the first layer of mesophyll cells.

In summary, the guard and epidermal cells of *P. leeanum* and other *Paphiopedilum spp.* have been shown to lack detectable levels of chlorophyll, whereas their mesophyll cells were chlorophyllous. The guard cells contained several mitochondria, leucoplasts and elaioplasts and lack anything resembling thykaloid membrane structure. The epidermal cells contained mainly etio-elaioplasts, and lacked starch. Each mesophyll cells contained several chloroplasts.

Several questions have been raised by this work some of which are fundamental to understanding stomatal mechanisms. Of particular interest are the following:

1. Is the nonchlorophyllous condition of the epidermis in *P. leeanum* limited to only *Paphiopedilum spp.?* A preliminary survey indicates that this condition is also present in *Cattleya, Coleogyne, Maxillaria, Renanthera* and *Suphronites spp.* (pers. comm., Dr. J. M. Mayo and A. Rencz). Possibly there is some taxonomic significance to these findings which warrants a more inclusive survey.
2. What is the cause of the non-chlorophyllous condition? Could it be the result of enzymatic and/or nutrient deficiencies?
3. What is the origin of guard cell starch and how similar are the guard and epidermal cell elaioplasts and etio-elaioplasts?

4. In terms of stomatal function, what role does the high guard cell concentration of starch, lipid and mitochondria play?
5. How dependent is the epidermis on the mesophyll, both metabolically and in terms of stomatal movements?
6. Do these non-chlorophyllous guard cells exhibit 'normal' stomatal responses? And, if so, what is the light receptor, is it contained in the epidermis or mesophyll?

Some of these questions will be dealt with in the following sections of this thesis, and more thoroughly discussed in the Conclusions.

PART 2 - PHYSIOLOGICAL INVESTIGATIONS

A. Introduction

Stomatal responses to light and CO_2 have been well documented (eg. Meidner and Mansfield, 1965 and 1968; Pallas, 1966; Levitt, 1967). 'Normal' responses can be considered to be of two types: those dependent upon CO_2 concentration, and those independent of it.

The CO_2 dependent response has been demonstrated by Heath and Russell (1954a), who found that decreasing external CO_2 concentration to the CO_2 compensation point caused an increase in stomatal aperture, and that an increase in CO_2 level above normal (ca. 300 ppm) caused a tendency to close. Heath and Russell (1954b) and Mansfield (1965) found that CO_2 free air in the dark caused the same degree of stomatal opening as in the light and normal $[CO_2]$. They found that the intercellular space CO_2 concentration was controlling the stomatal response to various CO_2 treatments. Light (eg., Meidner and Mansfield, 1968), temperature (eg., Heath and Orchard, 1957), and moisture stress (Heath and Meidner, 1961) have also been shown to influence stomatal movements by their effect upon intercellular space CO_2 levels.

Stomatal responses independent of CO_2 concentration have been demonstrated by Kuiper (1964), Heath, Mansfield and Meidner (1965), and Mouravieff (1958).

They found that blue light was more effective in causing stomatal opening than red light, on a quantum basis. Mansfield and Meidner (1966) were able to show that this occurred when the intercellular space CO_2 concentration was kept constant. Mouravieff (1974) has detected greater guard cell starch disappearance in 'blue' *vs* 'red' light. However, it is not understood how 'blue' light causes a greater stomatal opening. Meidner and Mansfield (1968) have posited the question as to the involvement of guard cell chlorophyll in the CO_2 independent response and concluded that the available evidence is inconclusive. CO_2 concentration (Gaastra, 1959), length of previous dark period (Mansfield and Heath, 1963), temperature (Meidner and Heath, 1959) and leaf water content (Stålfelt, 1955; Meidner, 1965) are also known to effect the CO_2 independent stomatal responses.

Therefore, 'normal' stomatal responses of non-Crassulacean Acid Metabolism (CAM) plants can be considered as follows:

1) Stomata open in the light and close in the dark.
2) Stomata tend to open in CO_2 free air and tend to close in high CO_2 air (greater than 300 ppm).
3) Blue wavelengths of light are more effective on a quantum basis than red, in causing stomatal opening.

Any theory explaining the mechanism of guard cell action must, as a minimum, account for these 'normal' responses.

Stomatal responses to various environmental factors can be measured by a variety of methods. The most common method is to measure the microscopic changes in stomatal pore aperture, and more recently, to measure the change in leaf diffusive resistance to water vapor. Variations of these and other techniques are summarized elsewhere (Heath and Mansfield, 1969; Meidner and Mansfield, 1968; Stigter, 1972). The diffusive resistance porometer of Kanemasu, Thurtell, and Tanner (1969) measures the diffusive resistance of a 1x2 cm leaf area. The technique allows rapid non-injurious measurements to be made of a large number of stomata. Diffusive leaf resistance (Rl) has been defined (eg. Nobel 1974) as follows:

$$Rl = \frac{(Rias + Rst)(Rc)}{Rias + Rst + Rc}$$

where:

> Rias is the resistance to water vapor diffusion offered by the intercellular air space in the leaf; Rc is the resistance offered by the cuticle; Rst is the resistance offered by the stomata, which is a function of stomatal pore aperture.

Rias and Rc are relatively constant components of leaf resistance, whereas, Rst is a variable component. Thus, changes in leaf diffusive resistance are indicative of changes in stomatal diffusive resistance, and therefore, changes in stomatal pore aperture (Kanemasu and Tanner, 1969a; Meidner and Mansfield, 1968). Typical values for open stomata are: crop plants, 1-5 sec/cm; mesophytes, 2-10 sec/cm; and xerophytes 5-20 sec/cm (Nobel, 1974).

The intact leaf experiments in this section were undertaken to determine whether or not *P. leeanum* exhibited 'normal' stomatal responses. The technique of the diffusive resistance porometer was employed rather than microscopic determination of changes in stomatal pore aperture, as the cuticular overlip (p. 25) made this measurement difficult and only semi-quantitative.

B. Materials and Methods

1. Plant growth conditions

The plants used for these experiments were maintained in the Botany Greenhouses, until needed, under the following conditions: 14-16 hour light period, constant temperature of 24 °C, relative humidity of 70-80%, and 20 changes of air per hour. Supplemental light was supplied by 400 W lucalux and multivapor lamps.

Studies were conducted in controlled environment chambers (Environmental Growth Chamber Inc. of Chargin Falls, Ohio - Models M3-65 and M11-41). Plants were transferred to growth chambers at least one month prior to their use in experiments, and kept under the following conditions: 14-16 hour light period, constant temperature of either 20, 24 or 26 °C, and relative humidity of 70-80%. Temperature and relative humidity were controlled to within 1 °C and ± 5% R.H. respectively. The light system of chamber model M11-41 consisted of either 0, 18, 42, or

60, 1000 W cool white fluorescent tubes and 0, 8, 16, or 24, 100 W incadescent bulbs. The light system of chamber model M3-65 was composed of either 0, 1, 2, or 3, 400 W lucalox lamps; 0, 1, 2, or 3, 400 W multivapor lamps; and 0, 2, 4, or 6, 150 W incandescent bulbs. A removable plexiglass barrier separated the lights from the chamber proper.

Plants were potted in 6 inch pots. The potting medium consisted of a loam-peat-sand mixture, sterilized sphagnum, and fir bark plus osmunda fiber (in portions of 1:1:4 by volume). Plants were divided when necessary, repotted, and fertilized with 1/10th strength Hoagland solution at approximately 12 month intervals.

2. Instrumentation

A Lambda, Model LI-185 Quantum/Radiometer/Photometer (Lambda Instruments Co., Lincoln Nebraska USA) was used to measure quantum flux (photosynthetically active radiation between 400 and 700 nm), solar radiation (global radiation from sun and sky, received on a horizontal plane), and illumination (as related to the CIE standard observer curve). An ISCO (Instrument Specialities Co., Lincoln Nebraska USA) spectroradiometer was used to measure the spectral distribution under various light sources (calibrated with an ISCO Spectroradiometer Calibrator).. Unless otherwise stated, the sensors were placed at leaf level perpendicular to the light source.

Leaf diffusive resistance to water vapor was determined with a Lambda Diffusive Resistance Meter Model LI-60 and Sensor Model LI-15S (Kanemasu et al., 1969). The sensor was calibrated as described in the Lambda manual, using calibration plates of known diffusive resistance. The sensor was cycled (dry to wet, then to dry) 10 times in preparation for use. When possible, calibration was carried out under experimental conditions. Leaf diffusive resistances were calculated from a temperature corrected calibration curve (in order to avoid error due to changes in leaf temperature). The leaf temperature was measured with the thermistor sensor located on the diffusive resistance sensor. The thermistor was calibrated and periodically checked against a 5 mil copper-constantan thermocouple. The thermocouple was mounted around the thermistor and connected to a Fluke Model 845 Ab Microvoltmeter with an Omega Model CJ-T Cold Junction Compensator

Relative humidity was measured with either wet and dry bulb thermocouples (5 mil) or paired diodes. Air flow past the sensors was achieved by a Reciprotor piston pump Type 506R (Reciprotor Ltd., Copenhagen Denmark) in the case of the thermocouples, and a Pamotor axial fan Model 8500C (Pamotor Inc., Burlingame Cal. USA) in the case of the diodes. Wind speed was measured with a Hastings Air Meter Model FB-27, unidirectional probe (Hastings-Raydist Inc., Hampton Virginia USA).

3. Light-dark responses

Two series of experiments were carried out: one in growth chamber model M3-65, and the other in model M11-41. Instrumentation and plant growth conditions were as previously described.

In both experiments, plants were placed in a plexiglass box (66x47x33 cm) within the growth chamber. Building air (ca. 300 ppm CO_2) was passed through an 8 liter distilled water bottle and into the box at a flow of 10 l/min. (measured with a Gilmont Size #3 Flowmeter F1300). Air circulation was achieved with a Pamotor axial fan Model 8500C. Relative humidity was maintained at 70-80%, and the chamber temperature was held constant.

For experiments done in chamber model M11-41, various light levels were achieved by changing the fluorescent and incandescent combination. A 1000W quartzline lamp (General Electric lamp with an Environmental Growth Chamber Inc., fixture) mounted above the plants provided additional lighting. The height of the lamp over the plants was adjustable.

Various combinations of chamber lights were employed for the experiments in chamber model M3-65. Seven mature leaves were used in this series of experiments.

Leaf diffusive resistances were measured in the dark, approximately one half-hour before the lights came on. Light stimulated changes in leaf diffusive resistance were monitored throughout the light period. Both adaxial and abaxial surface leaf diffusive resistance were measured.

4. Spectral and CO_2 concentration responses

Experimental conditions were similar to those employed in the previous section. The 1000 W quartzline lamp was used as the light source for experiments carried out in chamber model M11-41. Blue and red sheets of cellophane were placed between the light and the plexiglass box. The height of the lamp over the box was varied in order to approximate the light energy in both treatments.

For experiments done in chamber model M3-65, the chamber light system provided sufficient light energy so as not to require supplementation. Blue (Rohm and Haas 2424) or red (Rohm and Haas 2423) plexiglass barriers were placed between the lights and the chamber in order to illuminate the whole plant with either color.

Light treatment involved taking the plants from the dark condition to light of either color. Leaf resistances were measured in the dark, and then, throughout the light period. The leaves employed in these experiments were the same as those used in the light-dark response experiments.

CO_2 concentration experiments were carried out in both chamber models M3-65 and M11-41. High CO_2 treatment was maintained by using bottled gas of 1150 ppm CO_2. Low

CO_2 levels were developed by passing building air through 1 N KOH (treatment times were one hour). Building air (ca. 300 ppm) was used as normal CO_2.

C. Results

1. Light-dark responses

The adaxial surface leaf resistance (Rl) to diffusion of water vapor (adaxial Rl) was very high and relatively constant in both the light and dark (Table 2). Adaxial Rl was always greater than 100 sec/cm and significant light stimulated decreases in Rl were not detected. Adaxial Rl varied not only between leaves but also between halves of the same leaf (see leaf 3, sides a and b, Table 2). In most cases, the adaxial Rl approached the leak rate of the sensor (the measured resistance when the sensor is closed, minus a leaf, and placed in the same environment as the leaves).

The abaxial surface leaf resistance (abaxial Rl) was not constant. Abaxial Rl in the dark was generally greater than 50 sec/cm (Tables 2 and 3) and less than adaxial Rl (Table 2). Variation between leaves (Table 3), between halves of the same leaf (Table 2), and within the same leaf over time (Table 3), were noted for abaxial Rl of leaves in the dark.

Light stimulated decreases in abaxial Rl (Table 2 and Fig. 19) were observed for all leaves studied. The decrease in abaxial Rl in response to light was rapid;

TABLE 2 - Abaxial and adaxial leaf resistance of *P. leeanum*, in the dark and over time after lights on. Experiments done in chamber model M11-41, using four different leaves (each side of midrib was measured). Leaf temperature 21 C, R.H. 70-80%, light level 60 uE/m^2/sec. The numbering of these leaves is not comparable to that of other experiments done in chamber model M11-41.

Condition	Diffusive resistance sec/cm Dark		60 min. light		150 min. light		360 min. light		Ave.
Leaf Surface	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial
Leaf No.									
1a	302.2	288.1	253.1	36.0	211.4	31.4	237.5	25.4	251.1
1b	195.9	238.4	206.2	71.6	208.8	54.9	211.4	45.5	205.1
2a	207.7	147.5	212.7	40.3	185.3	21.5	206.2	20.3	203.0
2b	216.0	23.9	237.5	30.7	221.8	29.8	253.2	23.6	232.1
3a	288.1	269.2	260.9	45.5	186.6	29.8	232.3	24.9	242.0
3b	122.7	73.1	151.4	22.0	125.3	19.7	126.6	17.3	131.5
4a	281.0	314.0	237.5	45.5	216.6	22.0	249.2	17.3	246.1
4b	257.3	53.0	331.4	23.1	242.7	16.8	315.8	16.8	286.9
Ave.	233.8	175.9	236.3	29.0	199.9	23.9	229.7	23.9	224.8
Leak rate of sensor	-		274.2		208.8		313.2		

TABLE 3 - Abaxial leaf resistance of *P. leeanum*, in the dark one half hour before the lights came on. (a) same seven leaves over time in chamber model M3-65, same length of dark period prior to measurement, leaf temperature 19-21 C, R.H. 70-80%. (b) different seven leaves than in 'a', same leaves over time in chamber model M11-41, same length of dark period prior to measurement, leaf temperature 21-24 C, R.H. 70-80%.

(a)

Date Leaf	Abaxial leaf resistance sec/cm June 17	June 21	June 25	July 10	July 16	Ave.
1	257.9	50.3	88.7	377.5	137.0	182.3
2	462.6	139.1	83.9	32.5	32.9	150.2
3	397.5	-	83.9	61.5	116.6	164.9
4	794.9	381.5	275.9	88.5	46.8	317.5
5	28.7	343.1	285.5	161.3	123.1	188.4
6	383.6	127.1	172.7	102.5	190.5	195.3
7	230.1	105.5	56.1	70.5	116.1	115.7

Ave. 187.8

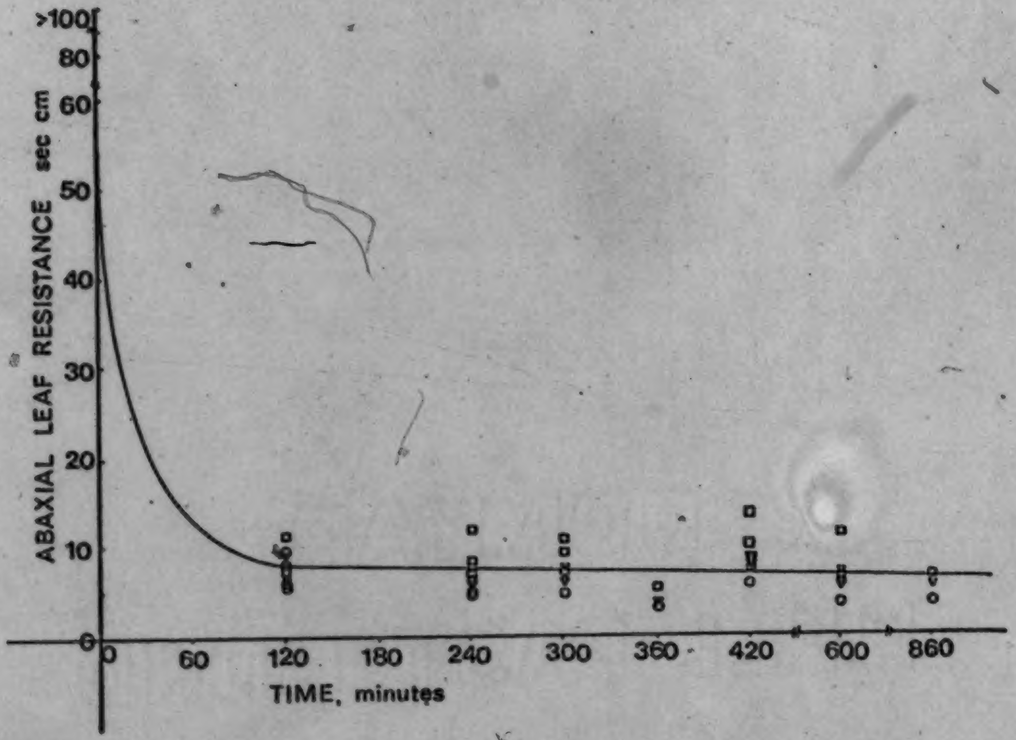
(b)

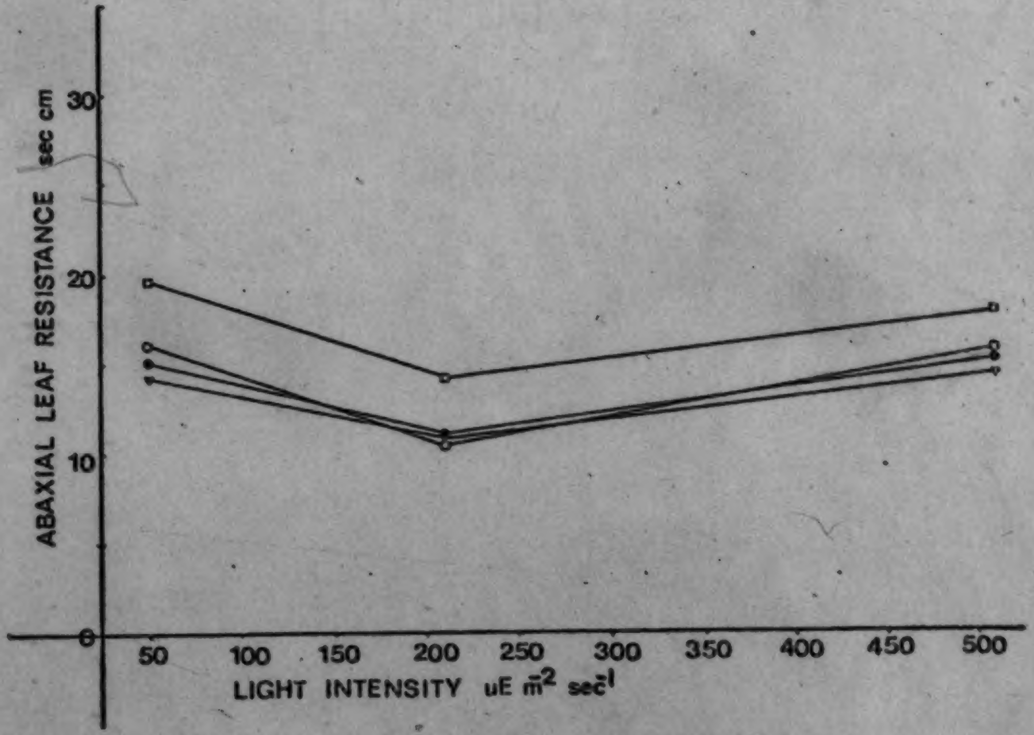
	Jan. 4	Jan. 5	Jan. 6	Ave.
1	368.2	197.5	628.8	398.2
2	35.3	210.8	274.8	173.6
3	62.9	189.5	290.8	181.1
4	222.2	242.8	408.2	291.1
5	795.4	413.5	349.5	519.5
6	192.2	90.8	114.8	132.6
7	20.4	31.6	86.8	46.3

Ave. 248.9

Figure 19. Abaxial leaf resistance of *P. leeanum*, over time since lights on in chamber model M11-41. Three different leaves on a number of different days at light intensities of 28, 33, and 125 uE/m^2/sec. Leaf temperature 21 C, normal CO_2, R.H. 70-80%.

Figure 20. Abaxial leaf resistance of *P. leeanum vs* light quanta above the plant in chamber model M3-65. Seven leaves measured at times of 60 min □; 120 min ▽; 180 min ○; since the lights were turned on. ● represents the average of all readings taken between 120 and 180 min after the lights come on. Leaf temperature 21 C, R.H. 70-80%, normal CO_2.


>100
80
60
50
40
30
20
10
0
ABAXIAL LEAF RESISTANCE sec cm
0
60
120
180
240
300
360
420
600
860
TIME, minutes


30
20
10
0
ABAXIAL LEAF RESISTANCE sec cm
50
100
150
200
250
300
350
400
450
500
LIGHT INTENSITY uE m^{-2} sec^{-1}

average changes greater than 80 sec/cm in less than 30 min. were measured (Table 4). Stable Rl values occurred (Fig. 19) and were maintained throughout light periods as long as 860 min, with slight variation (Fig. 19). Variations in abaxial Rl between leaves (Table 4), between halves of the same leaf (Table 2), and within the same leaf over time were noted. Generally, abaxial Rl for a leaf in white light equilibrated at a value less than 30 sec/cm and greater than 5 sec/cm (Tables 4 and 5). The mean abaxial Rl for all stable readings of the seven leaves used in chamber model M3-65 was 13.9 sec/cm (Table 5).

Saturation of light stimulated decreases in abaxial Rl occurred between 10 and 50 $uE/m^2/sec$ - measured above the plant (Figs. 20 and 21). Light readings were taken directly above the plant, and incident to either the abaxial or adaxial leaf surface. In terms of abaxial light levels, saturation may have occurred at less than 10 $uE/m^2/sec$ (Fig. 21).

Changes from light to dark stimulated increases in abaxial Rl in all leaves that were measured (Table 4). The increase in abaxial Rl in response to darkness was rapid. Average changes of greater than 100 sec/cm occurred in less than 15 min (Table 4). Leaf to leaf variations in the magnitude of response were noted.

TABLE 4 - Abaxial leaf resistance of *P. leeanum*, demonstrating light and dark responses, and the variation between leaves. Measurements on the same seven leaves in chamber model M3-65. Leaf temperature 20 C, R.H. 70-80%, light level 210 uE/m^2/sec above the plant.

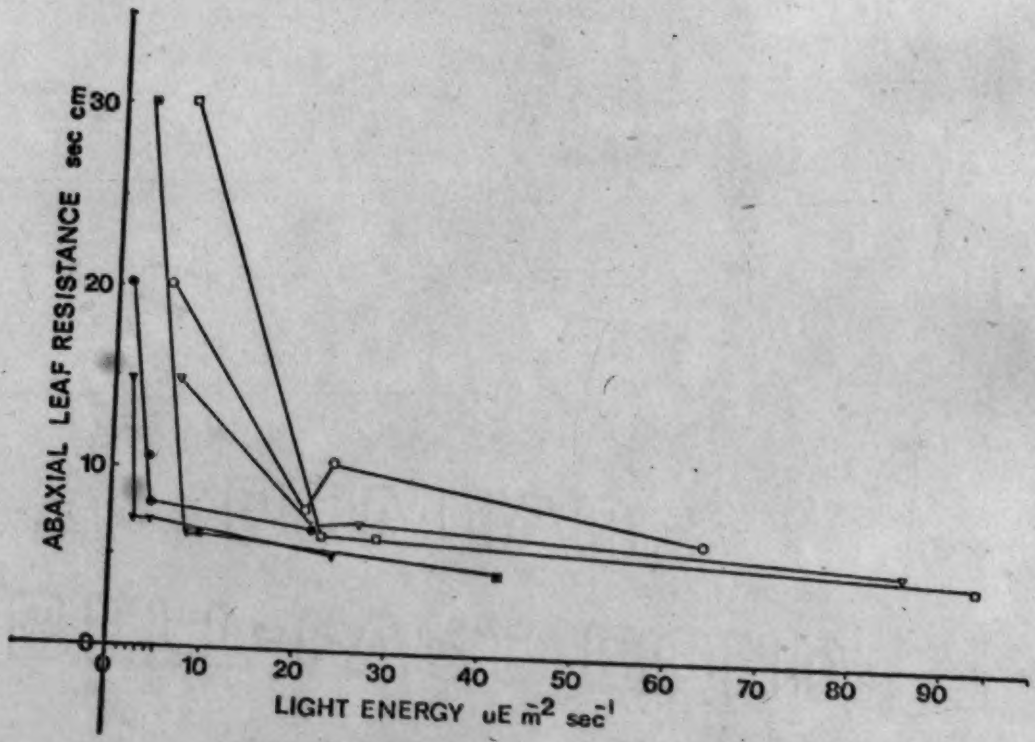
Leaf	Dark	Light 15 min.	Abaxial leaf resistance sec/cm 60 min.	120 min.	180 min.	240 min.	Dark 15 min.
1	137.0	11.6	11.5	11.3	12.3	12.7	164.6
2	21.9	7.4	6.5	5.6	6.2	7.6	76.3
3	116.6	32.9	5.4	6.7	5.3	6.1	48.1
4	46.8	11.7	11.7	10.8	10.7	10.2	141.8
5	123.1	30.4	22.3	13.5	10.7	13.2	157.0
6	190.5	38.3	24.8	14.8	13.6	15.9	402.6
7	116.1	21.6	16.2	13.1	12.4	13.7	111.4
Ave.	108.9	22.0	14.1	10.8	10.2	11.4	157.4

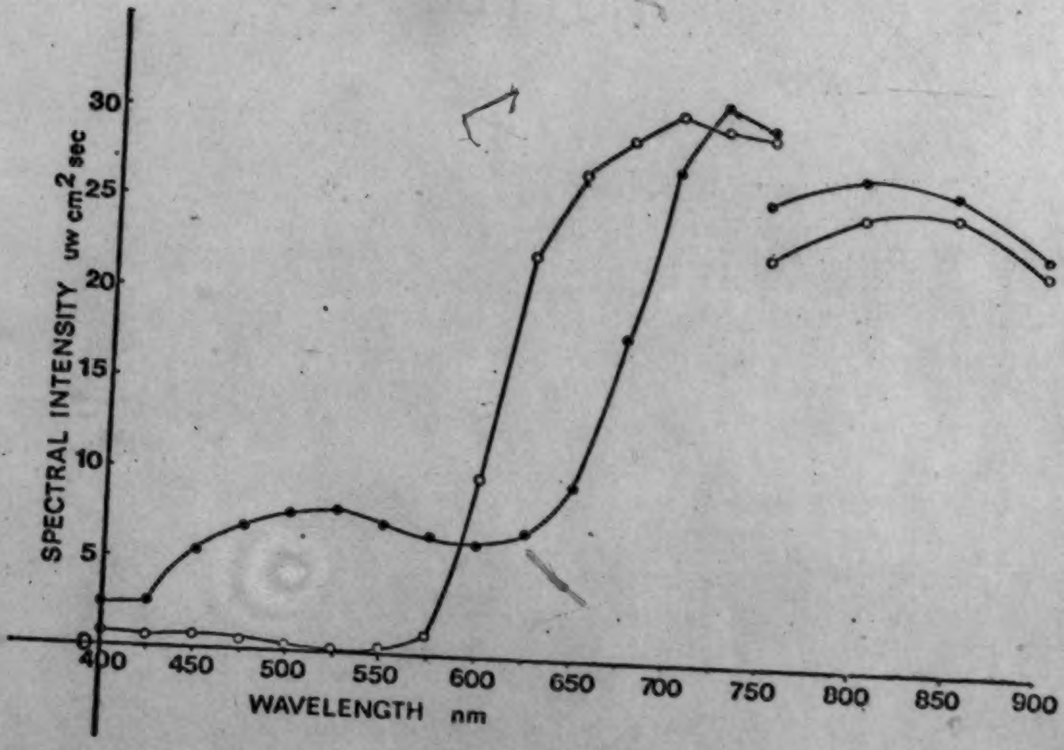
TABLE 5 - Abaxial leaf resistance of *P. leeanum*, statistical analysis of all readings on each leaf in chamber model M3-65, under various light treatments. All readings between 2 and 4 hours since lights on were used. Leaf temperature 21-24 C, R.H. 70-80%, all light treatments greater than 50 uE/m^2/sec.

	Abaxial leaf resistance sec/cm						
Leaf	White mean	Blue mean	Red mean	Paired-Variate Analysis of all readings on the seven leaves. Consecutive days paired			
1	13.3	16.2	23.3		White vs blue	White vs red	Blue vs red
2	7.2	7.1	12.4				
3	6.3	7.3	12.8	t	3.0	8.6	6.3
4	16.2	14.6	28.9	n	42	35	35
5	13.3	21.9	32.4				
6	20.7	24.9	39.2				
7	17.6	20.4	27.7				
Ave.	13.9	16.1	25.2				

Figure 21. Abaxial leaf resistance of *P. leeanum vs* light quanta incident to the adaxial (open points) and abaxial (solid points) leaf surfaces in chamber model M11-41. Three different leaves. All readings taken after 120 min since lights on. Leaf temperature 21 C, R.H. 70-80%, normal CO_2.

Figure 22. Spectral distribution of blue rich and red rich light treatments in chamber model M11-41. Total quanta of photosynthetically active radiation (PAR) in blue rich 135 uE/m^2/sec ●, in red rich 138 uE/m^2/sec o.


ABAXIAL LEAF RESISTANCE sec cm
30
20
10
0
0
10
20
30
40
50
60
70
80
90
LIGHT ENERGY uE m̄2 sec̄1

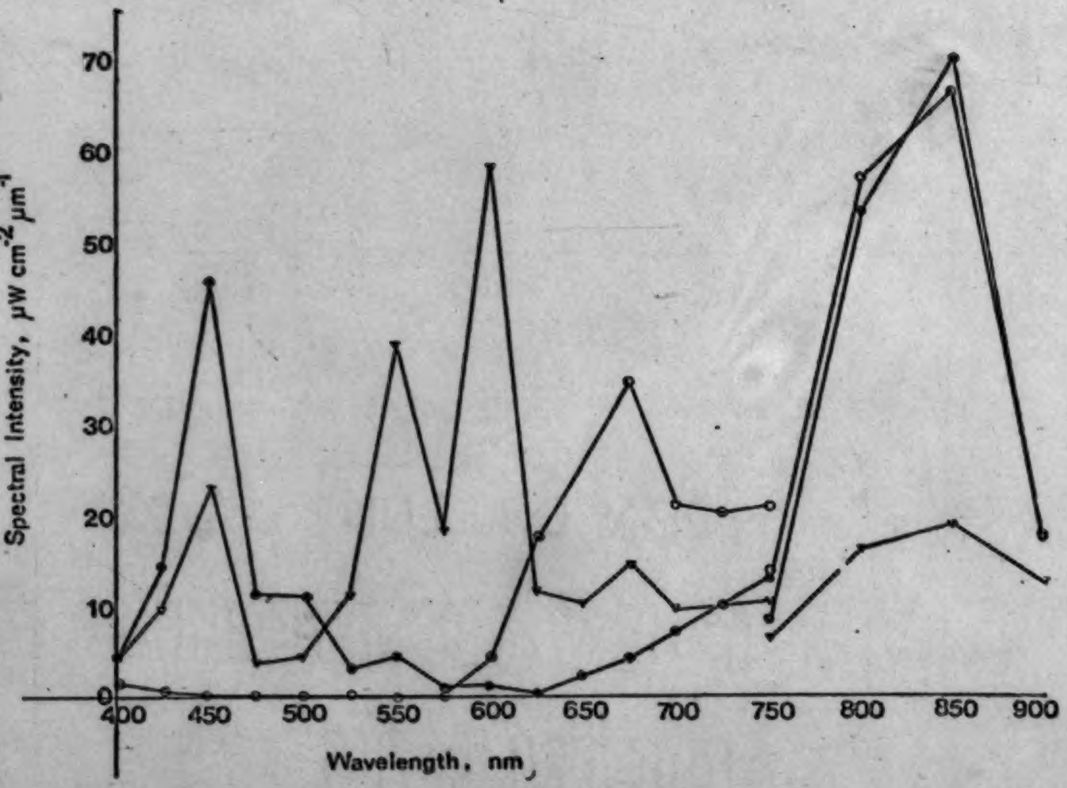

SPECTRAL INTENSITY uw cm2 sec
30
25
20
15
10
5
0
400
450
500
550
600
650
700
750
800
850
900
WAVELENGTH nm

2. Spectral and CO_2 concentration responses

The spectral analysis of the light systems used in the growth chambers models M11-41 and M3-65 are shown in Figs. 22 and 23. In both cases, a sharp spectrum was not obtained, however, each treatment consisted of greater amounts of blue or red wavelengths. The most precise spectrum was obtained in chamber model M3-65 where the colored plexiglass barriers were used.

When leaves were taken from the dark to light, blue-rich, red-poor light stimulated greater change in abaxial Rl than similar quanta of red-rich, blue-poor wavelengths (Tables 5 and 6). In the case of experiments done in chamber model M11-41, the average difference between blue and red treatments decreased with time until it was 1.5 sec/cm (Table 6a), whereas, in chamber M3-65 the difference remained evident with time (Table 6b). Paired variate analysis (Table 6b) of the data for each leaf under the two treatments (blue vs red light) indicates that, in the case of experiments done in chamber model M3-65, the difference between blue and red was significant at the 0.001 level. The same analysis of data from experiments done in chamber model M11-41 (Table 6b) indicates that the difference between the treatments was less significant than for experiments done in chamber model M3-65. For all experiments done in chamber model M3-65, the blue light treatment was significantly

Figure 23. Spectral distribution of blue rich and red rich light treatments in chamber model M3-65. Total quanta (PAR), in blue 100 uE/m^2/sec ●; in red 125 uE/m^2/sec ○; in white 215 uE/m^2/sec ▽ .



Spectral Intensity, μW cm⁻² μm⁻¹
0
10
20
30
40
50
60
70
400
450
500
550
600
650
700
750
800
850
900
Wavelength, nm

TABLE 6 - Abaxial leaf resistance, *P. leeanum*, blue and red light treatments on consecutive days. (a) same seven leaves in blue and red treatments over time since lights on, chamber model M11-41, leaf temperature 25 C, R.H. 70-80%, normal CO_2 air. (b) same seven leaves in blue and red treatments over time since lights on but different from those used in 'a', chamber model M3-65, leaf temperature 21 C, R.H. 70-80%, normal CO_2 air.

Time since lights on	Abaxial leaf resistance sec/cm							
	30 or 60 min.		120 min.		180 min.		260 min.	
Light treatment	blue	red	blue	red	blue	red	blue	red
Leaf								
(a)								
1	19.6	18.5	8.4	10.7	7.1	8.0	5.1	6.3
2	16.2	27.8	9.1	13.9	10.5	19.0	6.6	9.2
3	26.1	32.2	14.5	19.5	11.4	19.6	9.9	11.8
4	6.7	12.9	4.4	6.0	4.3	5.9	3.4	4.0
5	8.5	26.9	4.9	5.3	4.7	5.1	4.9	4.8
6	13.6	21.8	10.2	16.7	9.5	9.8	6.0	7.8
7	7.9	11.6	6.8	9.5	5.9	7.1	4.0	5.5
Ave.	14.1	21.7	8.3	11.5	7.6	10.6	5.7	7.2
Ave. difference	7.6		3.3		3.0		1.5	
Paired variate analysis significantly different at	.02-.01		.01-.002		.10-.05		.01-.002	
(b)								
1	21.6	39.2	15.9	25.9	16.2	22.9		
2	10.9	21.8	6.7	13.8	6.6	12.5		
3	11.1	19.2	8.8	17.7	5.8	9.5		
4	19.7	40.0	14.3	36.4	13.8	21.6		
5	42.1	65.8	21.9	36.9	17.3	29.3		
6	37.5	58.5	24.7	34.1	21.6	37.3		
7	29.8	25.2	21.6	30.4	17.8	24.9		
Ave.	24.4	30.5	16.3	27.9	14.2	22.6		
Ave. difference	10.5		11.6		8.4			
Paired variate analysis significantly different at	.001		.001		.001			

TABLE 7 - Abaxial leaf resistance, *P. leeanum*, high, low, and normal CO_2 treatment of leaves in red and blue light treatments. (a) same seven leaves on consecutive days in chamber model M3-65, leaf temperature 21 C, R.H. 70-80%, treatment times one hour (b) same seven leaves, different from 'a' in chamber model M11-41, leaf temperature 25 C, R.H. 70-80%, treatment times one hour.

	Abaxial leaf resistance sec/cm							Paired variate analysis of difference between CO_2 treatments	
CO_2 treatment	Low CO_2		Normal CO_2		High CO_2				
Light treatment	blue	red	blue	red	blue	red		blue	red
Leaf (a)									
1	12.6	23.2	13.8	25.9	27.0	46.8	Normal CO_2		
2	7.8	8.4	6.5	9.8	13.6	20.0	vs. high CO_2	.01-.002	.01<.002
3	6.5	8.5	5.8	14.2	16.1	19.5			
4	10.9	13.8	11.8	16.8	16.3	29.6	Normal CO_2		
5	15.5	23.7	16.0	26.4	37.7	48.9	vs. low CO_2	<.10	<.05
6	18.4	28.0	16.1	33.9	43.6	64.5			
7	14.6	22.7	17.3	18.9	26.4	55.4	High CO_2		
Ave.	14.1	17.1	12.5	20.9	25.8	40.7	vs. low CO_2	.01-.002	.002-.001
Red vs Blue statistically different at	.02-.01		.02-.01		.02-.01				
(b)									
1	4.7	6.4	-	-	17.3	21.3	Low vs high		
2	5.7	10.5			15.6	21.8	CO_2	.001	.001
3	8.4	16.8			28.1	38.3			
4	3.2	6.3			9.7	15.1			
5	3.6	6.1			12.3	17.9			
6	5.4	7.1			17.3	21.3			
7	3.6	6.0			8.8	12.2			
Ave.	4.9	8.5			15.6	21.1			
Red vs Blue statistically different at	.001				.001				

different from the red light treatment at the 0.001 level (Table 5).

In the light, high CO_2 treatment for 60 min tended to cause increases in abaxial Rl (Table 7). High CO_2 stimulation of increases in abaxial Rl was always significant at the 0.01 level when compared to normal CO_2 treatment, and at the 0.001 level when compared to the low CO_2 treatment. Low CO_2 treatments in the light generally caused decreases in abaxial Rl, but these decreases were not significantly different at the 0.05 level when compared to normal CO_2 treatments. The CO_2 response occurred in both the red and the blue light treatments (Table 7). Results with low CO_2 treatment of leaves in the dark were inconclusive as an effect was not shown.

D. Discussion

The resistance to diffusion of water vapor through the adaxial leaf surface of *P. leeanum* is high and relatively constant in that, it is always greater than 100 sec/cm, and, no light stimulated changes in adaxial Rl are detectable. As this surface lacks guard cells (p. 21), the adaxial Rl is approximately equal to adaxial Rc. Nobel (1974) states Rc values ranging from 30 to 200 sec/cm, and Milthorpe (1969), 50 to 400 sec/cm, as common for a variety of plants. Using a similar measurement technique as used here, Sharpe (1973) found the adaxial Rl for a hypostomatous *Eucalyptus* species to never be less than

300 sec/cm. The average adaxial Rl or Rc for 8 leaf surfaces of *P. leeanum* is 225 sec/cm (Table 2). Although the physical and chemical nature of the adaxial leaf surface cuticle is unknown (shown to be more important in determining Rc than cuticle thickness - Kamp, 1930), these resistance values fall within the stated ranges. Variations in adaxial Rl or Rc between halves of the same leaf and between leaves are partially due to localized differences in the cuticles' chemical and physical nature. Of special importance is the wax component (Horrocks, 1964), the extrusion of which increases with age (Holmgren, Jarvis and Jarvis, 1965), and the arrangement of wax platelets (Martin and Juniper, 1970). Care should be taken in interpreting the variations and actual values of adaxial Rl or Rc reported here, as these resistance values are in a range in which the linearity of the sensor has not been tested, and very often approximate the leak rate of the sensor.

Although Rc is known to be affected by leaf temperature (Holmgren et al., 1965), leaf water status (Milthorpe, 1960), and relative humidity (Moreshet, 1970), Rc as well as Rias are considered to be relatively constant (Nobel, 1974). As a result, any distinct change in Rl of a leaf surface containing guard cells is due to changes in Rst (Kanemasu and Tanner, 1969a; Meidner and Mansfield, 1968). Changes in Rst are the direct result of changes in stomatal pore aperture (Nobel, 1974). As the abaxial leaf surface of *P. leeanum* contains guard cells (p. 21), a change in abaxial Rl is the result of change in stomatal aperture.

In the dark, the abaxial Rl for *P. leeanum* is relatively high and is generally greater than 50 sec/cm. The average of all abaxial Rl readings is greater than 100 sec/cm. Kanemasu and Tanner (1969b) defined Rl greater than 100 sec/cm as indicating complete stomatal closure in *Phaseolus vulgaris* (complete opening equalled 4 sec/cm). When stomata are completely closed Rl is approximately equal to Rc (Ehler and Van Bavel, 1968). Thus, the Rc of the average abaxial leaf surface of *P. leeanum* is probably greater than 100 sec/cm. Rl values less than this most likely indicate incomplete stomatal closure, and possibly, localized variation in Rc. Evidence from light and electron microscopy (size, shape, and staining properties) indicate that the abaxial and adaxial cuticles are similar. Thus, the differences between adaxial Rl and abaxial Rl in the dark may be due to the lack of complete stomatal closure.

In leaves of *P. leeanum*, light stimulates decreases in abaxial Rl and thereby increases in stomatal aperture. Sawyer (1932) reported sluggish or no stomatal response to light for *Vaccinium macrocarpon*, a species which is also reported to lack guard cell chlorophyll but contains a normal chlorophyllous mesophyll. Stomatal opening in *P. leeanum* is not at all sluggish (average changes of greater than 80 sec/cm in 30 min). The time for and the extent of stomatal opening induced by light after a dark period is known to vary, between species (Meidner and Mansfield, 1968), with the state of 'readiness to open' (Stålfelt, 1927), with light intensity (Virgin, 1956), CO_2 concentration (Gaastra, 1959), and the length of the previous dark period (Mansfield and Heath, 1963). In these experiments, the previous dark period was maintained at the same length and daily period to insure similar states of 'readiness to open'. Typically, stomatal opening starts within minutes and is complete within 35 min for abaxial stomata of *Vicia faba* (Kassam, 1973), 90 min for stomata in tobacco leaf discs (Zelitch, 1961), and between 120 and 150 min for stomata of *Xanthium pennsylvanicum* (Mansfield and Heath, 1963). Light induced stomatal opening in *P. leeanum* is typical in that it starts within minutes and is completed between 60 and 120 min. Opening over time follows a sigmoid curve, as found for a variety of other species (Gregory and Pearse, 1937). The equilibration values are maintained over time indicating the absence of mid-day closure under growth chamber conditions and the lack of a dominant endogenous rhythm causing oscillations in stomatal aperture. It is doubtful that by coincidence all measurements were made during a similar point of the rhythm.

Stomatal closure in response to darkness in *P. leeanum* is rapid. The average change of greater than 100 sec/cm in less than 15 min approximates the amount of time required for stomatal closure in other species such as *Nicotiania tabacum* (Zelitch, 1961), *Phaseolus vulgaris* (Kanemasu and Tanner, 1969b), and *Xanthium pennsylvanicum* (Mansfield and Meidner, 1966).

Saturation of the light response for guard cells on the abaxial leaf surface of *P. leeanum* occurs between 10 and 50 uE/m^2/sec (light measured above plant). Kanemasu and Tanner (1969b) indicate that it is the light that reaches the guard cells (sum of incident abaxial illumination and transmitted light through the mesophyll reaching the guard cells) that is important in determining the light saturation level. This light level was not determined. However, light levels incident to the abixial surface indicate that saturation of the stomatal response to light in *P. leeanum* may occur at light levels lower than 10 uE/m^2/sec. Generally, higher light levels are required to saturate the stomatal light response of a variety of species. Kanemasu and Tanner (1969b) found light saturation of *Phaseolus vulgaris* stomata to occur at 10 uE/m^2/sec (total abaxial illumination). Kassam (1973) found light saturation of abaxial stomata of *Vicia faba* to be 40 uE/m^2/sec (incident to the abaxial surface). Relative to these crop plants, *P. leeanum* could very well be considered as a shade plant in that it has low light level requirements for saturation of its stomatal response to light.

The overall quanta measure (PAR) for the 'blue' and 'red' light treatment in chamber model M11-41 was approximately equal. However, the spectral analysis indicates that the actual amount of quanta in blue wavelengths (425-490 nm) was considerably less than that in red wavelengths (640-740). Also the 'blue' treatment contained considerable amounts of red wavelengths. Haberman (1973) indicates that wavelengths other than red and blue have a differential effect upon stomatal opening. She implies that there are two photoreactions operating in stomatal opening; a low intensity response determined by green and far red wavelengths, and a high intensity response due to the red and blue portions of the spectrum. Therefore, the results obtained with *P. leeanum* in chamber model M11-41 may involve differences in stomatal opening not specific to red and blue wavelengths. The greater initial amount of stomatal opening in 'blue' may be partially due to greater amounts of green wavelengths (490-560 nm) causing the low intensity stomatal responses in so far as red wavelengths were approximately equal in the 'red' and 'blue' treatments. The decrease in the difference between 'red' and 'blue' treatments with time is possibly the result of there being greater quanta of red wavelengths in the 'red' treatment than blue wavelengths in the 'blue' treatment. Therefore, the greater effectiveness of similar quanta of blue over red wavelengths could be expressed by the consistent difference between the 'blue' and 'red' treatments. The greater effectiveness of blue over red wavelengths is more clearly shown in experiments done in chamber model M3-65. In this case, the treatments contained similar quanta of blue and red wavelengths without too much overlap in the rest of the spectrum (excluding far red). Not only was the initial rate of opening greater in blue, but the difference between red and blue was maintained over time. Mansfield and Meidner (1966), and Meidner (1968) reported a six to seven fold greater effectiveness of blue over red for *Xanthium pennsylvanicum.* In *P. leeanum,* the difference between blue and red is less than this, but always significantly different. Other workers, Meidner (1968) and Mouravieff (1958), have reported differences in effectiveness of blue over red as low as 3X for *Allium cepa* and *Veronica beccabunga,* and as high as 17X for *V. beccabunga.* The difference between these species and *P. leeanum* is probably due to measurement technique and the ability to control light quality. These workers were able to use sharp bands of treatment wavelength, whereas, the treatments used here were only relative amounts of blue *vs* red (i.e. blue rich-red poor, or red rich-blue poor).

Variations in the effectiveness of blue over red wavelengths of light have been reported to occur as the light level is changed from less than light saturation to saturation levels (Hsiao, Allaway and Evans, 1973; Mouravieff, 1958). The experiments with *P. leeanum* were done at light levels greater than saturation, the less than saturation stomatal response characteristics to red and blue light treatments are unknown. The length of previous dark period (Mansfield and Heath, 1963) and temperature (Meidner and Heath, 1959; Mansfield, 1965) are known to effect stomatal spectral responses. In these experiments, both were maintained constant in the 'red' and 'blue' treatments. Haberman (1973) reported a significant phytochrome effect on the stomata of *Helianthus annuus*, whereas, Evans and Allaway (1972), Hsiao et al. (1973), and Mansfield and Meidner (1966) did not detect a phytochrome response in stomata of *Vicia faba* and *Xanthium pennsylvanicum*. Stomatal response to phytochrome in *P. leeanum* remains undetermined. The significant difference between the 'blue' and 'white' light treatments at saturating light intensities, confirms the CO_2 independence of the 'blue' light response.

A CO_2 free air effect in the dark on the stomata of *P. leeanum* was not shown. Heath and Russell (1954b), and Meidner (1965) found that it was the CO_2 concentration of the intercellular air space that affected stomatal responses. Heath (1950), and Scarth and Shaw (1951) have shown that if stomata are completely closed, CO_2 free air has no effect on stomata. The high abaxial Rl (indicating closed stomata), and the impermeability of cuticle to CO_2 (Holmgren et al., 1965) partially explain the lack of a CO_2 free air response in the dark for the stomata of *P. leeanum*. Further experimentation is needed in order to determine whether or not the above is the case in *P. leeanum*. High CO_2 treatment in the light causes stomatal closure in leaves of *P. leeanum*. As the stomata were open, the CO_2 treatment was able to effect changes in the intercellular air space CO_2 concentration and thereby, stomatal aperture. Stomata did not completely close as a result of high CO_2 treatment in the light. Presumably a change in darkness is required before complete closure occurs (Heath and Russell, 1954b). A significant low CO_2 effect on stomata of *P. leeanum* as compared to normal CO_2 in the light did not occur. Meidner and Mansfield (1968) report that in most C3 species, stomatal aperture increases as CO_2 treatment decreases from normal CO_2 (ca. 300 ppm) to 100 ppm CO_2, changes below 100 ppm CO_2 have no further effect. In *P. leeanum*, the relatively high Rl in the light could allow the intercellular CO_2 concentration to be depleted by mesophyllic photosynthesis to levels in the neighborhood of 100 ppm CO_2 under normal CO_2 treatment conditions. The low CO_2 treatment would allow further reduction in the intercellular space CO_2 concentration, but as these reductions would be below 100 ppm CO_2, there would be no effect on stomatal aperture. This, then, could explain the lack of a significant effect of low CO_2 treatment in the light on the stomata of *P. leeanum*.

In summary, the stomata of *P. leeanum* have been shown to exhibit the 'normal' stomatal responses, in that they: open in the light, close in the dark at normal rates; respond to CO_2 concentration changes (close with high CO_2 treatment in the light); and, open more readily when treated with similar quanta of blue vs red wavelengths of light. These findings show that normal stomatal responses, at least in *P. leeanum* and possibly other species, are not dependent upon the presence of guard cell chlorophyll. The significance of these findings is discussed in the Conclusions.

PART 3 - POTASSIUM ANALYSIS

A. Introduction

In the recent literature, there is considerable evidence to suggest that potassium ion (K^+) flux between guard cells and subsidiary and/or epidermal cells plays a key role in turgor operated stomatal movements (Hsiao, 1975). As stomata open, K^+ accumulation in guard cells has been shown to occur in osmotically significant amounts in *Commelina communis, Nicotiania tabacum, Vicia faba,* and *Zea mays.* Under conditions known to produce stomatal opening, the presence of potassium in guard cells has been demonstrated to occur in a large number of species (Dayanandan and Kaufman, 1973; Willmer and Pallas, 1973).

An analysis of the potassium concentration in the leaf of *P. leeanum* was undertaken in order to determine if potassium was involved in its stomatal operations.

B. Materials and Methods

1. Potassium stain

The 'K stain' of Macallum (1905) was used to detect potassium in the guard and epidermal cells of *P. leeanum, P. insigne* and *V. faba.* The reagent used was sodium cobaltinitrite. It forms a yellow triple salt precipitate with potassium, and when stained with ammonium sulphide, forms a black sulphide precipitate which can easily be seen under the microscope.

The stain was prepared according to Allaway and Hsiao (1973) - 27.5 g of sodium cobaltinitrite was placed in 38 ml of distilled water plus 5 ml glacial acetic acid, and made to a total volume of 55 ml with distilled water. Building air was bubbled through the solution to insure saturation. The stain was decanted off and stored in a cold refrigerator. The staining and washing times were varied; in all cases the following staining sequence was employed:

1. wash epidermal peel in distilled water (to rid peel of mesophyllic debris)
2. wash in 0.1 mM CaCl2 (to maintain membrane integrity and to rid peel of mesophyllic debris)
3. stain with prepared stain (forms yellow precipitate with K^+ and indicates stain penetration)
4. wash in distilled water (to rid epidermis of excess stain which reacts with ammonium sulphide to form a black coloration)
5. stain with ammonium sulphide (forms black precipitate with yellow potassium precipitate)

All solutions were kept in an ice bath, and on occasion, a drop of Triton X was added to the 'K stain' to aid stain penetration.

The abaxial leaf epidermis was stripped from intact leaves which had been kept in the light for more than 2 hours. Also, the abaxial epidermis was stripped from detached leaves which had been kept in a petri containing KOH pellets for a CO_2 free air treatment. *V. faba* and *Z. mays* were used as controls, as they have been reported to accumulate sufficient K^+ to show detectable changes in guard cell potassium (Fischer, 1972; Pallaghy, 1971).

2. Flame photometery and atomic absorption spectrophotometery

Stripped abaxial epidermal peels and their underlying mesophyll plus adaxial epidermis were washed in 4 changes of 0.1 mM $CaCl_2$ and then distilled water, total washing time of 11 min, dried for 24 hours at 100 °C, and then weighed. Total extractable potassium (via perchloric acid) was determined on the two samples. As a result of the necessity to collect an extremely large epidermal sample, in which contamination from adhering mesophyll was noticed, the more sensitive technique of atomic absorption spectrophotometry was also employed.

Potassium concentrations were determined using a Perkin-Elmer Atomic Absorption Spectrophotometer Model 305B (A.A.), with a P.E. HGA-2000 Controller for the graphite furnace and a Deuterium Arc Supply. A Westinghouse K hollow cathode lamp was used as the light source (supplied by Canlab, catalogue number S7275-19). The A.A. was set-up under the following conditions: 'Dry' temperature of 100 °C for 30 sec, 'Charr' temperature of 1200 °C for 15 sec, 'Atomizing' temperature of 1900 °C for 15 sec, wavelength of absorption - 383.2 nm, and slit width of 5. Calibration curves were constructed from solutions prepared from a 1000 ppm KCL standard (Harleco, Phil., Pa. 19143 USA). Each time the machine was used, calibration curves were constructed and then the tissue was analysed. All glassware, pipettes, slides and forceps were washed in 50% HNO_3 and then thoroughly washed in distilled water from the same source as that used in making the calibration solutions. This same distilled water served as the blank during calibration.

Sections of abaxial epidermal strips, from leaves that had been in the light for at least 3 hours, were washed for 5-10 min in 0.1 mM $CaCl_2$ and then distilled water for 30 sec. The strips were oven dried at 100 °C for 24 hours. Test sections other than those used in the potassium determinations were stained prior to drying with concentrated neutral red (.05%) in order to determine relative epidermal cell intactness. Sections of epidermis were analysed after area and number of cell determinations.

C. Results

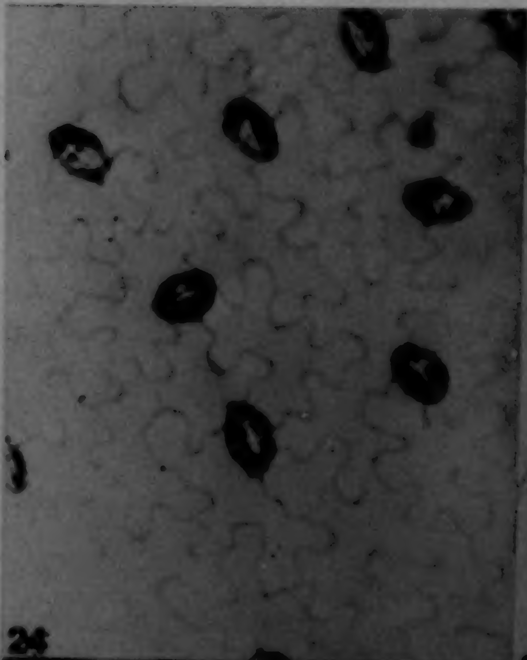
1. Potassium stain

Guard cells on the abaxial epidermal peels from *V. faba* leaf sections which had been kept in light and CO_2 free air for three hours showed detectable amounts of potassium when stained with the 'K stain' (Figs. 24 and 25). The precipitate was black and distinct. On occasion, precipitate bodies aggregated together. The amount of precipitate varied from none per guard cell to

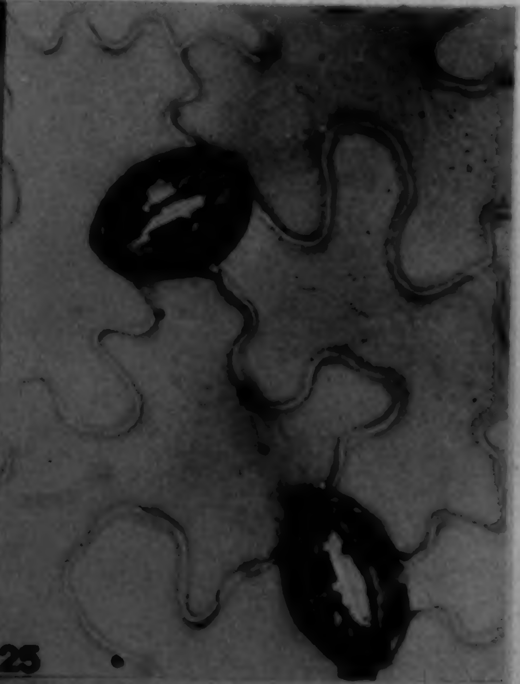
Figure 24. Bright field micrograph, *V. faba* , abaxial epidermis stained with sodium cobaltinitrite. Stomata were in the open condition prior to staining which causes the stomata to close. X 256.

Figure 25. Bright field micrograph, *V. faba*, abaxial epidermis stained with sodium cobaltinitrite. Stomata in open condition prior to staining. X 640.

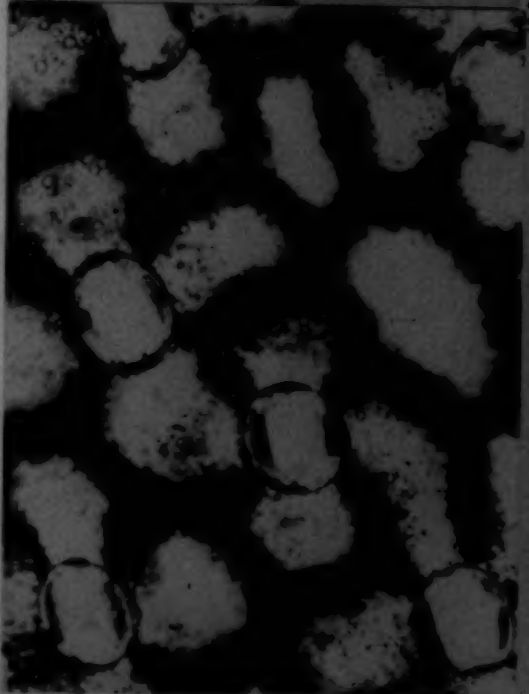
Figure 26. Bright field micrograph, *P. leeanum*, abaxial epidermis stained with sodium cobaltinitrite. Abaxial Rl prior to stripping, 11.5 sec/cm; treatment conditions of light and CO_2 free air. X 256.



24



25



completely filled guard cells, however, these were the exception. Average amounts of potassium precipitate per guard cell are represented in Figs. 24 and 25. Epidermal cells of *V. faba* generally lacked potassium precipitate, however, the occasional cell was noticed to contain considerable amounts. The peeling process broke most of the epidermal cells of *V. faba*.

Under the same treatment conditions and using the same 'K stain' and procedure, the guard cells of *P. leeanum* and *P. insigne* were found to lack potassium. Precipitate was rarely observed in the guard cells of these species. The times for each step of the staining procedure were varied, but similar results were obtained. The original wash in distilled water was varied from 10 sec to 2 min, the wash in 0.1 mM $CaCl_2$ from 2 min to 30 min, the stain in sodium cobaltinitrite from 10 sec to 120 min, the second wash in distilled water from 5 to 45 min, and the stain in ammonium sulphide from 1 to 120 min. Evidence of stain penetration was noted as the guard cell cytoplasm and nucleus were stained yellow (color of dilute stain) after these treatment times.

The epidermal cells of *P. leeanum* generally did not contain any potassium precipitate (Fig. 26), however, the epidermal cells of *P. insigne* did contain some precipitate. The attached mesophyll of these species contained considerable amounts of potassium precipitate, and as a result the peels had to be floated rather than immersed in the treatment solutions (floating mesophyllic debris became trapped in the cuticular suprastomatal chamber and obscured results). The epidermal cell walls of *P. leeanum* and *P. insigne* showed different staining reactions compared with the cell walls of *V. faba*. The cell walls of *V. faba* did not react with the stain, whereas, those of *Paphiopedilum spp.* did (Figs. 24, 25, and 26). However, in *Paphiopedilum spp.* the stain did not form a precipitate. The darkened central portion of each guard cell of *P. leeanum* (Fig. 26) was seen to be the result of the underlying epidermal cell wall, which had taken up the stain.

2. Flame photometery and atomic absorption spectrophotometry

The flame photometry mesophyll sample (mesophyll plus adaxial epidermis) consisted of 1.4073 g dry weight, with an adaxial surface area of 172.6 cm^2. When analysed for potassium, the total extractable potassium in this sample was found to be 1.867% on a dry weight basis, or 2.626×10^{-2} g (Table 8).

Over 70 collections of abaxial epidermal peels were required to collect 0.5847 g dry weight of epidermis, with a surface area of 565.9 cm^2. This sample when analysed with the flame photometer contained 0.054% potassium on a dry weight basis, or 3.187×10^{-4} g (Table 8). Some contamination of the epidermal sample by mesophyllic

TABLE 8 a - Flame photometery analysis for potassium in the abaxial epidermis and in the mesophyll plus adaxial epidermis of *P. leeanum*.

	Dry weight g	Area cm^2	Potassium analysis % dry wt.	Potassium concentration uEq g^{-1}	pEq* mm^{-2}
Abaxial epidermis	.5847	565.97	.0545	13.97	144.37
Mesophyll plus adaxial epidermis	1.4073	172.59	1.867	478.46	38,924.3

b - If it is assumed that all the potassium from analysis is in the guard cells and that there are 51.9 guard cell per mm^2 and 11.7 p l per guard cell (see table 10)

	K concentration g per l of guard cell volume	Eq per l of guard cell volume
Abaxial epidermis	9.27	.238

c - Grams of mesophyll contamination necessary to account for all the potassium in the abaxial epidermis of *P. leeanum* = 17.1 mg.

* - p = 10^{-12}
Eq = equivalents

debris was noticed (this was unavoidable due to the large amount of tissue required for the sample). As a result, the epidermal potassium concentration was thought to be high and the atomic absorption spectrophotometery technique was employed because much smaller samples were needed. It would only have taken 17.1 mg of mesophyll attached to the total epidermal sample to account for all the potassium (Table 8).

The A.A. calibration curves were constructed using KCl solutions of 0.005, 0.01, 0.02, and 0.05 ppm, and are shown in Fig. 27. Testing with more concentrated solutions indicated that the curve became nonlinear at approximately 0.2 ppm. Data from Dec. 16 and Jan. 29 are included; other experiments were designed to determine appropriate sample size and handling techniques. The most suitable sample size was found to be in the range of 0.15 to 0.30 mm^2 of epidermis, much smaller than that needed for flame photometry.

The A.A. analysis results and calculations are shown in Tables 9 and 10. Assuming that all the potassium from the analysis was contained in guard cells and that the average guard cell volume was 11.7 picoliter (pl), the mean concentration of potassium in a single guard cell was found to be 64.4 pg, 1.65 pEq, or 0.141 Eq/l. Assuming that potassium was evenly distributed between all epidermal cells including guard cells and that the

Figure 27. Calibration curves for Atomic Absorption Spectrophotometery determination of potassium. Blank values were determined from the distilled water used to make up the calibration solutions, and were subtracted from the values for each solution to give the curves. Dec. 5; Dec. 16; Jan. 29.

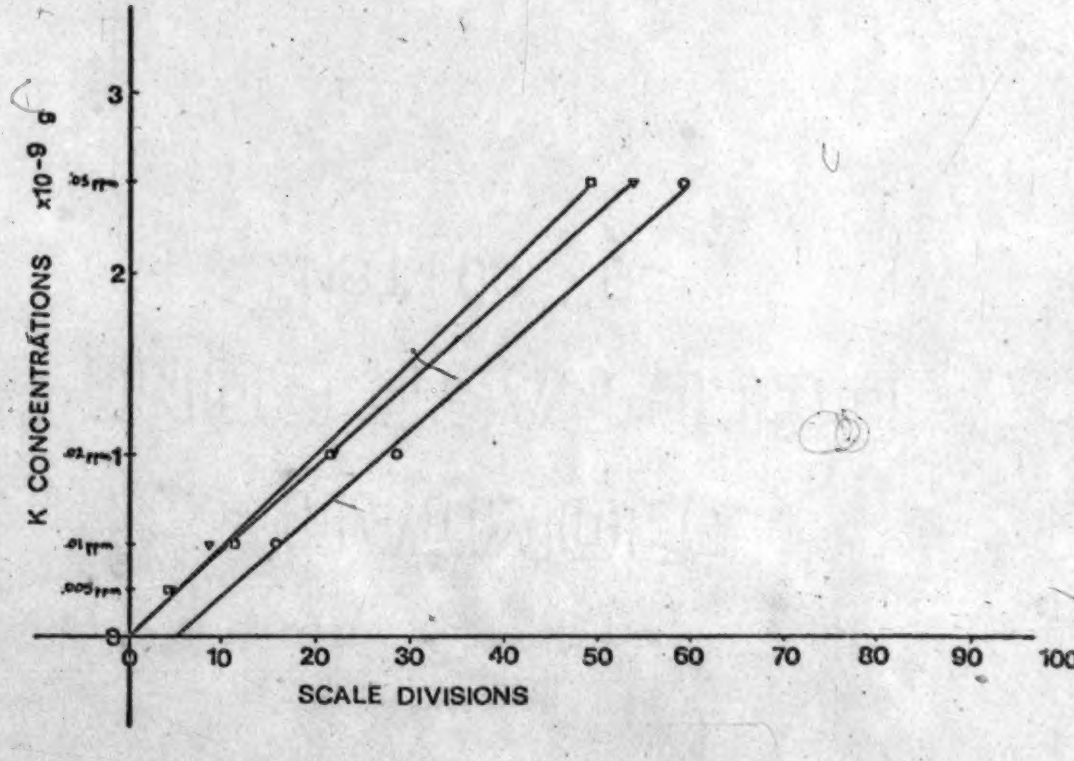

K CONCENTRATIONS x10-9 g
3
.05 ppm
2
.02 ppm 1
.01 ppm
.005 ppm
0
0
10
20
30
40
50
60
70
80
90
100
SCALE DIVISIONS

TABLE 9 - Atomic Absorption (A.A.) analysis for potassium in the abaxial epidermis of *P. leeanum.* Sample size and number of guard cells measured directly. % intact epidermal cells measured from neutral red staining. Each number is an average of three.

Jan. 29	Number guard cells	Sample size mm^2	Potassium analysis x 10^{-9}g	% intact epidermal cells
1	14	.29	1.05	73
2	14	.24	.55	64
3	16	.26	1.40	58
4	16	.29	1.18	79
5	17	.29	.83	80
6	9	.18	.45	81
7	5	.14	.43	72
8	11	.22	.44	
9	11	.21	.62	
10	19	.30	1.75	
11	10	.24	.60	
Dec. 16				
1	70	-	4.8	
2	72	-	3.0	
3	76	-	3.2	
4	66	-	3.1	

TABLE 10 - The concentration of potassium in the epidermis of *P. leeanum*, as calculated from Atomic Absorption (A.A.) analysis*, Table 9. Values are mean concentrations, at 95% level.

1) All potassium in guard cells

Number samples	Potassium per guard cell pg	pEq	Eq l^{-1}
11	64.4±12.8	1.65±.33	.14±.02

2) Potassium evenly distributed

Number samples	Potassium per cell with 100% intact epi.		Potassium per cell with 72% intact epi. cell	
11	.054±.013	.0014±.0003	.074±.018	.0019±.0002

3) Amount potassium on dry weight of epidermis basis

Number samples	% Potassium	uEq g^{-1}
15	.032±.005	8.18±1.29

*The above assumes the following for calculation purposes

a. guard cell volume is approximately equal to
(Lx.5WxD)+ (Lxπ (.5W)^2) = 11.7 pl
L = length, D = depth
W = width of cylinderical portion

b. epidermal cell volume is approximately equal to
LxWxD = 385.6 pl
L = W

c. 51.9 guard cells per mm^2, L = 67.5 um, W = 14.3 um
D = 1.7 um

d. 161 epidermal cells per mm^2, L = W = 75.3 um
D = 68 um

average epidermal cell volume was 385.6 pl, the mean concentration of potassium was 0.054 g/l or 0.0014 Eq/l. Neutral red staining of adjacent epidermal peels (Jan. 29) indicated that approximately 72.4% of the epidermal cells and 100% of the guard cells were intact (Table 9). If potassium was evenly distributed between intact cells, the concentration of potassium was 0.074 g/l or 0.0019 Eq/l (Table 10).

Based on the dry weight to area ratio found in the flame photometery sample and a guard cell count of 51.9 per mm^2, the percent of potassium in the epidermis on a dry weight basis was found to be 0.032% (Table 10). This represents approximately 8.18 uEq/g dry weight epidermis.

D. Discussion

The amount of potassium precipitate found in the guard cells of *V. faba* (Figs. 24 and 25) is similar to that reported elsewhere (Allaway and Hsiao, 1973; Fischer, 1971 and 1972) for open stomata. A comparison to photographic standards prepared by Fischer (1972) indicates that these stomata contain osmotically significant amounts of potassium in terms of stomatal operation. The general lack of epidermal cell potassium precipitate may be the result of epidermal cell damage during the peeling process (Fischer, 1972), or may be the result of low epidermal cell concentrations which are not detectable by the sodium cobaltinitrite test (Dayanandan and Kaufman, 1975;

Willmer and Pallas, 1974). The variations in amount of potassium precipitate per cell reported here were also noted by the above mentioned authors.

Although the stain and procedure were shown to work with *V. faba*, the presence of potassium in the guard cells of *P. leeanum* and *P. insigne* could not be demonstrated. Using this technique Willmer and Pallas (1974) had difficulty detecting potassium in the guard and epidermal cells of monocot species with elliptically shaped guard cells unless the stomata were open. The treatment conditions used, and the abaxial Rl values for *P. leeanum*, indicate that the stomata were open prior to stripping and staining for potassium. Stain penetration to the guard cell cytoplasm was shown by the yellow coloration after staining.

These results do not necessarily indicate a complete lack of potassium in the guard cells of *P. leeanum* and *P. insigne*, but do indicate less than osmotically significant amounts relative to *V. faba*. Most of the epidermal cells of *Paphiopedilum spp*. epidermal peels were intact, therefore, the lack of potassium precipitate is most likely due to low concentrations.

According to Macallum (1905), the stain precipitates potassium upon contact, therefore, dark coloration of cell walls in *P. leeanum* and *P. insigne* is interpreted as stain absorption not reaction with potassium. Obviously there is some difference in the cell wall properties between *V. faba* and *Paphiopedilum spp.*

Although the flame photometry analysis involved an extremely large sample size, the results contain important information. Willmer, Pallas, and Jackson (1974) reported that the potassium concentration on a dry weight basis for the mesophyll of *V. faba* and *C. communis* was 2.69% and 1.98% or 632 uEq/g and 481 uEq/g, respectively. These values, as well as the value reported here for *P. leeanum* (1.87% or 478 uEq/g), fall within the mean range (1.66 to 2.75%) reported for sixteen species by Evans and Sorger (1966). Thus, the mesophyll tissue of *P. leeanum* contains what could be considered normal amounts of potassium.

The concentration of potassium in the abaxial epidermis of *P. leeanum* is 0.05% or 14 uEq/g based on the flame photometery analysis and 0.03% or 8.2 uEq/g according to the A.A. analysis. It was felt that the flame photometery values were high due to attached mesophyllic debris, which the A.A. analysis avoided because only epidermal strips free of mesophyllic debris were analysed. The potassium concentrations from either analysis are considerably lower than normal. Willmer et al. (1974) report potassium concentrations (dry weight basis) of abaxial epidermis with open and closed stomata for *V. faba* and *C. communis* to be 4.38% for open to 4.41% for closed, and 3.09% for open to 3.02% for closed, or 997 to 1038 uEq/g and 731 to 711 uEq/g respectively. Not only is the potassium concentration in the abaxial epidermis of *P. leeanum* much less than these values, but also, it is much less than in the mesophyll on a dry weight basis. In *V. faba* and *C. communis*, the potassium concentration of the abaxial epidermis exceeds that of the mesophyll on a dry weight basis suggesting a process resulting in the accumulation of potassium, whereas in *P. leeanum*, the abaxial epidermis contains a much lower concentration of potassium than in the mesophyll suggesting a process which excludes potassium from the epidermis.

Based on the assumption (by no means warranted by the evidence) that all the abaxial epidermal potassium was contained in the guard cells of *P. leeanum*, the potassium content per guard cell would be 64.4 pg or 1.7 pEq (A.A. analysis). This amount of potassium is equal to a concentration of 0.14 Eq/l. Allaway (1973) and Allaway and Hsiao (1973) report guard cell potassium concentrations ranging from 0.11 to 0.15 Eq/l for closed stomata and 0.54 Eq/l for open stomata of *V. faba*.

The calculated concentration in the guard cells of *P. leeanum* is more similar to the closed concentrations for *V. faba*, however, the reported values for *V. faba*; are based on an analysis of an epidermis which did not contain any intact epidermal cells. In the case of *P. leeanum* , the epidermal sample contained on the average 72.4% intact cells. Potassium staining of intact epidermal cells when stomata are open indicates that in *V. faba* (Allaway and Hsiao, 1973; Fischer, 1972) and *C. communis* (Willmer and Pallas, 1973), there are considerable amounts of potassium in these cells. Also the work of Pearson (1975) with *C. communis* shows that only 25±10% of total epidermal potassium migrates into guard cells upon stomatal opening. Thus the assumption that all the epidermal potassium is contained within guard cells of *P. leeanum* is a poor one. Also, the assumption that all the potassium was in the guard cells of *P. leeanum* is incorrect as the estimated concentration should have reacted with the potassium stain as in the case of Allaway and Hsiao (1973) and suggests a more even distribution between guard and epidermal cells. Based on even distribution of potassium in the epidermis of *P. leeanum*, the concentration of potassium is 0.0014 Eq/l for 100% intact cells or 0.0019 Eq/l for 100% intact guard cells and 72.4% intact epidermal cells. The relative distribution of potassium between guard and epidermal cells in the epidermis of *P. leeanum* is unknown and could be determined with an electron probe analysis.

These results suggest that in *P. leeanum*, potassium ion is not the main osmoticum involved in stomatal movements. Other inorganic ions that could play a significant role are sodium and chloride.

CONCLUSIONS

Before discussing the questions raised by this work, the major findings will be restated. The anatomical investigations showed the following:

1) the guard and epidermal cells of *Paphiopedilum fairrieanum*, *P. harissanium*, *P. insigne*, and *P. leeanum* do not contain detectable levels of chlorophyll, whereas the mesophyll cells do contain 'normal' amounts.

2) mature guard, epidermal and mesophyll cells are cytoplasmically quite different. The guard cells contain mainly elaioplasts, leucoplasts and mitochondria; the epidermal cells contain mainly etio-elaioplasts; the mesophyll cells contain mainly chloroplasts with and without starch inclusions.

3) no guard cell to guard cell or guard cell to epidermal cell plasmodesmatal connections were seen, whereas the epidermal cells are interconnected by plasmodesmata. Epidermal to mesophyll cell plasmodesmata are present.

The physiological investigations showed the following:

1) the guard cells of *P. leeanum* respond to light and dark, CO_2 concentration and blue wavelengths of light are more effective than red in causing stomatal opening on a quantum basis.

2) stomatal responses to these environmental factors are not at all sluggish and approximate rates found in a variety of other species.

3) Abaxial leaf resistances in *P. leeanum* when stomata are closed are generally greater than 50 sec/cm, when stomata are open generally less than 30 sec/cm. Adaxial leaf resistance values are always greater than 100 sec/cm (this surface lacks stomata).

The potassium investigations showed the following:

1) the potassium concentration in the epidermis of *P. leeanum* is less than normal and is not present in sufficient concentrations so as to be involved osmotically in guard cell operations.

2) the mesophyll of *P. leeanum* contains normal amounts of potassium.

3) the results suggest that potassium is excluded from the epidermis of *P. leeanum* leaves.

These major findings, as discussed in their appropriate sections, command serious attention. Until the present, no species has been reported in which normal stomatal responses were exhibited by stomata lacking guard cell chlorophyll. Also until the present study, no species has been reported in which potassium was not the major osmoticum involved in stomatal operation. These findings suggest that in *P. leeanum*, a uniquely different stomatal mechanism may be in operation, or that, these components (chlorophyll and potassium) may not be essential to any stomatal mechanism.

Based on indirect evidence, the role of guard cell chlorophyll as an essential part of the opening process has been questioned previously (Meidner and Mansfield, p. 81, 1968). They point out that opening can occur in the dark with low or free $C0_2$ air treatment, and that high $C0_2$ treatment causes closure whereas the reverse would be expected if role of chlorophyll was through photosynthesis Conversely, there is considerable evidence to indicate that guard cells do not function unless they contain sufficient chlorophyll levels (see p. 1). For example, ontogenetic studies have shown that only stomata with chlorophyll will respond to light (Frommhold, 1971), and that, the occurrence of well developed chloroplasts in guard cells corresponds with the start of stomatal functioning (Reuter, 1942).

Presently, the proposed role of guard cell chlorophyll in stomatal opening is the production of ATP from Photosystem I. Humble and Hsiao (1969) have shown that Photosystem II is not essential. It has been suggested that this ATP would participate in potassium accumulation in guard cells (Pallas and Dilley, 1972). Pearson and Milthorpe (1974) imply that potassium accumulation and CO_2 fixation compete for the ATP, thus explaining the low CO_2 requirement for stomatal opening. The low CO_2 conditions would result in a guard cell cytoplasmic pH change favoring starch hydrolysis (Levitt, 1967) and subsequent production of organic acid anions for electrochemical balancing and H^+ ions for cation exchange with potassium (Levitt, 1974). In *P. leeanum,* the CO_2 dependent stomatal responses are obviously independent of guard cell chlorophyll. The low CO_2 requirement for stomatal opening could be provided for by mesophyllic photosynthesis resulting in reduced guard cell CO_2 concentrations, or by CO_2 fixation by enzymes such as PEP carboxylase. If an inorganic ion flux is involved in *P. leeanum* (could be tested for by electron probe analysis) the energy for ion uptake, if active, could come from mitochondrial activity as a result of starch hydrolysis and/or unsaturated lipids being fed into the tricarboxyic acid cycle, or from another pigment system not detected in the microscopic studies.

In terms of the $C0_2$ independent stomatal response, this work answers the question posited by Meidner and Mansfield (1968, p. 24), "Do the chloroplast pigments absorb the energy for the $C0_2$ independent opening reaction?". As guard cells lacking chloroplasts and chlorophyll exhibit these responses, the answer is obviously no. The mechanism by which blue wavelengths of light are more effective than red in causing stomatal opening (on a quantum basis) is unknown. Mouravieff (1974), using microphotometric measures of guard cell starch content, detected 3 to 4 times more starch disappearance in blue vs red wavelengths for a variety of species. Voskresenskaya (1972) reports that blue light enhances non-photosynthetic ^{14}C incorporation into glutamate, asparatate and malate at the expense of endogenous sucrose and sugar, as compared to red. As malate levels in guard cells are positively correlated to aperture (Allaway, 1973), the $C0_2$ independent response in *P. leeanum* and other species may be due to enhanced activity of the enzymes of starch hydrolysis and glycolysis, yielding malate.

Abscisic acid (ABA) is known to produce stomatal closure (Mittelheuser and Van Steveninck, 1969), prevent opening (Horton, 1971) and cause starch accumulation and K^+ efflux from guard cells (Mansfield and Jones, 1971). The site of ABA synthesis in response to water stress is in the chloroplast (Milborrow, 1974). As it has been assumed that guard cell chloroplasts are capable of ABA synthesis, it is of particular interest to speculate on the role of ABA in the stomatal responses of *P. leeanum*. Does ABA effect the stomatal complex of *P. leeanum* and if so, is the source of ABA the underlying mesophyll, thereby making stomatal responses of *P. leeanum* dependent upon the mesophyll?

The findings with *P. leeanum* raise the important question as to the degree of independence of the epidermist from the mesophyll. Chemical analysis, of metabolite exchange between the epidermis and mesophyll (Pearson, 1973) and of the enzymes in each (Willmer et al., 1973), indicate that the epidermis and mesophyll are relatively separated from each other in terms of stomatal operations. The lack of changes in total epidermal potassium as guard cells open in *Commelina* (Pearson, 1975) and occurrence of high concentrations of substances in the epidermis and not in the mesophyll, such as calcium oxalate crystals (Weber, 1955), support this conclusion. Some findings of this work, normal potassium concentrations in the mesophyll, but low potassium concentrations in the epidermis suggesting exclusion, further support this hypothesis. However, the presence of pit fields between epidermal and mesophyll cells in *P. leeanum*, and the fact that epidermal metabolism may ultimately depend on an external source of chemically fixed energy, suggest that at least some communication is possible. This could also be suggested by the lack of starch in the mesophyll adjacent to the epidermis. Excluding another pigment system in the guard cells of *P. leeanum* and a total dependence of stomatal responses on intercellular air space CO_2 determined by the mesophyll (for example the CO_2 independent responses), some communication between the mesophyll and guard cells is necessary in order to explain stomatal responses. Insight into the importance of the mesophyll to stomatal responses could be gained from experiments with isolated epidermal strips. For instance, do isolated epidermes display normal stomatal responses to light and CO_2, and if so, what are the necessary components of the supporting solution in order that normal responses are exhibited? Studies such as this have been undertaken, however the results to the present time are inconclusive.

The process whereby potassium is excluded from the epidermis, when normal amounts of mesophyllic potassium are present, commands serious inquiry. The concentration of potassium in the epidermis (dry weight basis) is below those reported by Evans and Sorger (1966) for leaves showing potassium deficiencies. It is interesting to note that a symptom of potassium deficiency is chlorosis and that potassium is required for the activity of δ-aminolevulinic dehydratase. This enzyme is involved in the synthesis of porphobilinogen, a precursor to chlorophyll formation (Rebeiz and Castelfranco, 1973). Although potassium deficiencies are known to affect the activity of many enzymes (Evans and Sorger, 1966 - Table IV), it may well be that the exclusion process producing a potassium deficient epidermis in *P. leeanum* is related to the non-chlorophyllous condition of this epidermis. In terms of noted changes in guard cell solute concentration during stomatal opening, another univalent cation such as Na^+ may function in place of potassium. Although Na^+ is known to substitute for K^+ in the activation of certain enzymes (Evans and Sorger, 1966), Na^+ does not substitute for K^+ in activating δ-aminolevulinic hydratase (Burnham and Lascelles, 1963).

In conclusion, this thesis has raised several interesting questions, and provided a few answers. Obviously there is much to be done before the mechanism of stomatal operation can be worked out. It may well be that a unique mechanism operates in *P. leeanum*, or that a new interpretation of stomatal research is required in light of these findings.

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APPENDIX A

Suppliers from which Paphiopedilum spp. were obtained are listed below:

Armacost and Royston Inc.
11920 La Grange Ave.
PO Box 25576
W. Los Angeles, California. P. leeanum

Penn. Valley Orchids
239 Old Gulph Road
Wynnewood, P.A.19096 P. insigne

A. Seidel
1981 Rua Roberto Seidel
1981 Cable Address Corupa
Santa Catarina, Brazil P. leeanum

Stewart Orchids Inc.
1212 E. Lastunas Drive
PO Box 307 San Gabriel, Calif. P. fairrieanum
P. leeanum